THE ROLE OF GAP JUNCTIONS IN ADRENAL GLAND FUNCTION

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ABSTRACT

This thesis explores the theory that gap junctions play a role in adrenocortical homeostasis and function. To test this hypothesis, gap junction mediated communication was investigated in the intact adrenal gland. In addition, to determine the impact of altered adrenal trophic state on gap junction distribution and expression, adrenal glands from hypophysectomized mice as well as normal and neoplastic human adrenal tissues were evaluated.

Gap junction mediated intercellular communication was done in primary mouse adrenal glands using a modified Lucifer Yellow dye communication assay. Dye communication showed that the adrenal cortex was communication competent and that functional cell-cell communication was directly related to the abundance of connexin 43 expression. There was an absence of dye communication in the outer cortical zona glomerulosa (ZG) that was contrasted by a high level of communication in the inner zones of the zonae fasciculata/reticularis (ZF/ZR).

The removal of the endogenous pituitary derived ACTH stimulus by hypophysectomy resulted in a significant loss of connexin 43 expression in the ZF/ZR that was associated with a diminished trophic state. The loss of connexin 43 expression was concurrent with features associated with a diminished functional state of the gland including loss of lipid droplets and reduced expression of the ZG specific cytochrome P_{450} aldosterone synthase (P_{450} aldo). A

comparison of adrenal glands from connexin 43 deficient mice (Cx 43 -/-) demonstrated several features that were similar to those observed in hypophysectomized mice, including glandular atrophy and reduced P_{450} aldo expression in the ZG.

Connexin 43 expression and distribution in the normal human adrenal gland was identical to that previously described in the adrenal glands from other mammals. Connexin 43 expression was abundant in the ZF/ZR and nearly absent from the parenchymal cells of the ZG. Immunocytochemial analysis of neoplastic tissues showed that benign (adenoma) and malignant (carcinoma) neoplasms lost 30% and 90% respectively, of their connexin 43 expression in the ZF/ZR when compared to normal tissues.

The data presented here demonstrate that connexin 43 expression is related to physiological changes that affect adrenal trophic state and suggest that gap junction mediated cell-cell communication is involved in the function of the adrenal cortex. Furthermore, because connexin 43 deficient animals also exhibit glandular changes consistent with a diminished state of the adrenal gland, it supports a role for proper gap junction expression in adrenal cortex development and function.

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1. CHAPTER 1

1.1. Introduction

Gap junctions are plasma membrane channels that facilitate the direct intercellular exchange of a number of small signaling and biologically relevant molecules. Although the presence of gap junctions has been extensively documented in multiple tissue types and implicated in development, differentiation (Kumar and Gilula, 1996; Nicholson and Bruzzone, 1997; Romanello et al., 2001; van der Heyden et al., 2000) and hormonal response (Schiller et al., 1992), the regulatory function of gap junction mediated communication remains a relatively unexplored phenomenon. This study seeks to better define gap junction mediated intercellular communication (GJIC) in the adrenal gland, a hormonally responsive tissue that must function differentially as a result of physiological demands. To that end, the hypothesis that gap junctions are dependent on the trophic state of the adrenal gland was tested. Adrenal gap junction dynamics were investigated to determine their influence on adrenal gland function. In addition, the potential role of gap junctions in maintaining normal adrenal functions was also investigated.

1.2. Adrenal gland

To fully appreciate the significance of the gap junction mediated intercellular communication between adrenocortical cells, it is first necessary to discuss basic adrenal morphology and physiology. The adrenal gland is an endocrine organ that is essential for life because it secretes several glucocorticoids and mineralocorticoids that regulate both metabolic and electrolyte levels. The adrenal gland can be subdivided into two embryologically distinct regions: the adrenal cortex and the adrenal medulla. The adrenal medulla, which arises from the

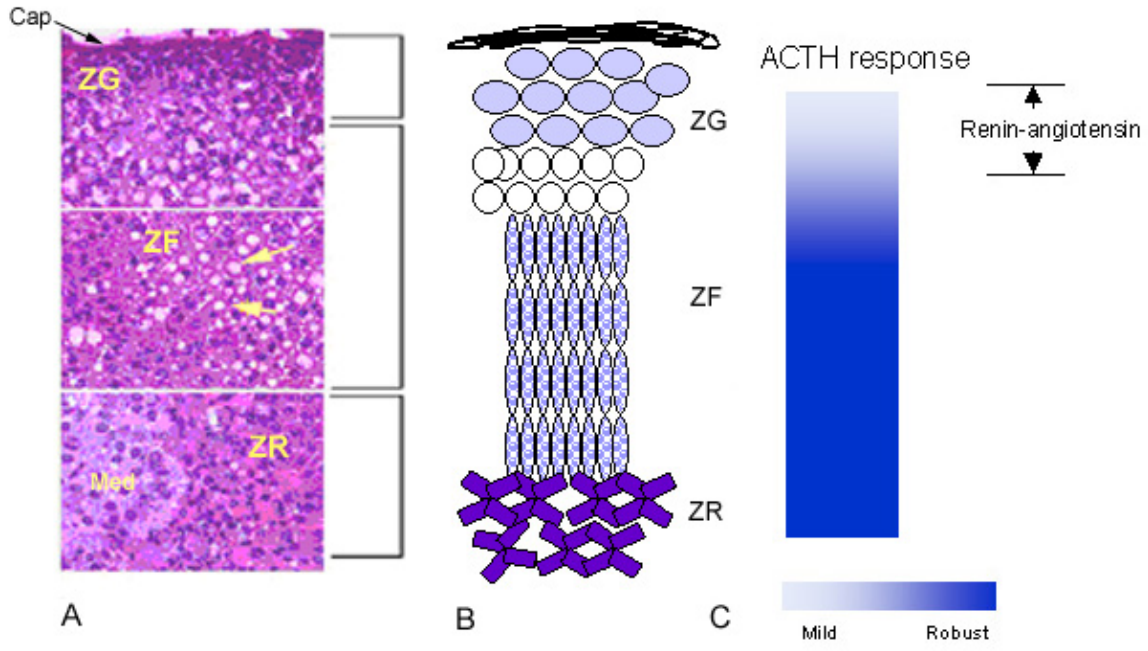

Figure 1-1 Adrenal gland and morphology and hormone responsiveness

Adrenal gland morphology is depicted in a hematoxylin/eosin stained specimen, which illustrates the diversity of the cells of the adrenal cortex. Beneath the adrenal capsule (Cap), are the three adrenal cortical zones: the zona glomerulosa (ZG), zona fasciculata (ZF), zona reticularis (ZR) and their distinct morphology is apparent. Note the especially high level of lipid droplets in the zona fasciculata (arrows, A). A diagrammatic depiction of adrenal morphology highlights the variations in zonal morphology and boundaries between the corical cells (B). Zonal differences are also reflected in the variations in hormonal response (C). The peptide hormone, ACTH has a variable effect within the adrenal cortex that is most pronounced in the ZF/ZR and minimal in the ZG where renin-angiotensin signaling predominates. Med = extracortical cells of the adrenal medulla.

neuroectodermal cells, is situated within the center of the gland and ultimately gives rise to both the norepinephrine/epinephrine secreting chromaffin cells and ganglia (James, 1992; Parmer, 2002). The adrenal cortex, which will be the focus hereafter, is mesoderm derived and comprises three concentric zones of anatomically and physiologically distinct cells: the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) (Fig. 1-1).

Morphological features that distinguish the three cortical zones are echoed in the physiological and steroidogenic differences in the cortical regions. The zona glomerulosa cells reside in the outermost zone of the adrenal cortex. These cells are organized into ovoid clusters of cells that are interrupted by the connective tissue trabeculae (Fig. 1-1). The cells of the zona glomerulosa secrete mineralocorticoids, primarily aldosterone, which regulates plasma sodium and potassium concentrations. Immediately beneath the zona glomerulosa is the zona fasciculata. The zona fasciculata is characterized by linear arrays of cells that produce glucocorticoids, such as corticosterone (rodents) or cortisol (humans) (Fig. 1-2). Glucocorticoid hormones are responsible for regulating an entire suite of genes necessary for gluconeogenesis and glucose metabolism in response to physiological stress (Ehrenreich, 1999; Ehrhart-Bornstein, 1998; Holmes, 2001). The third and innermost zone of the adrenal cortex is the zona reticularis (ZR). The zona reticularis is composed of the androgen producing (primarily dehydroepiandrosterone *DHEA*) cells that are arranged in a meshlike pattern and are located immediately beneath the cells of the zona fasciculata and abut the medullary cells (Rainey, 1999; Rainey, 2002).

Steroidogenesis in the Three Adrenal Cortical Zones

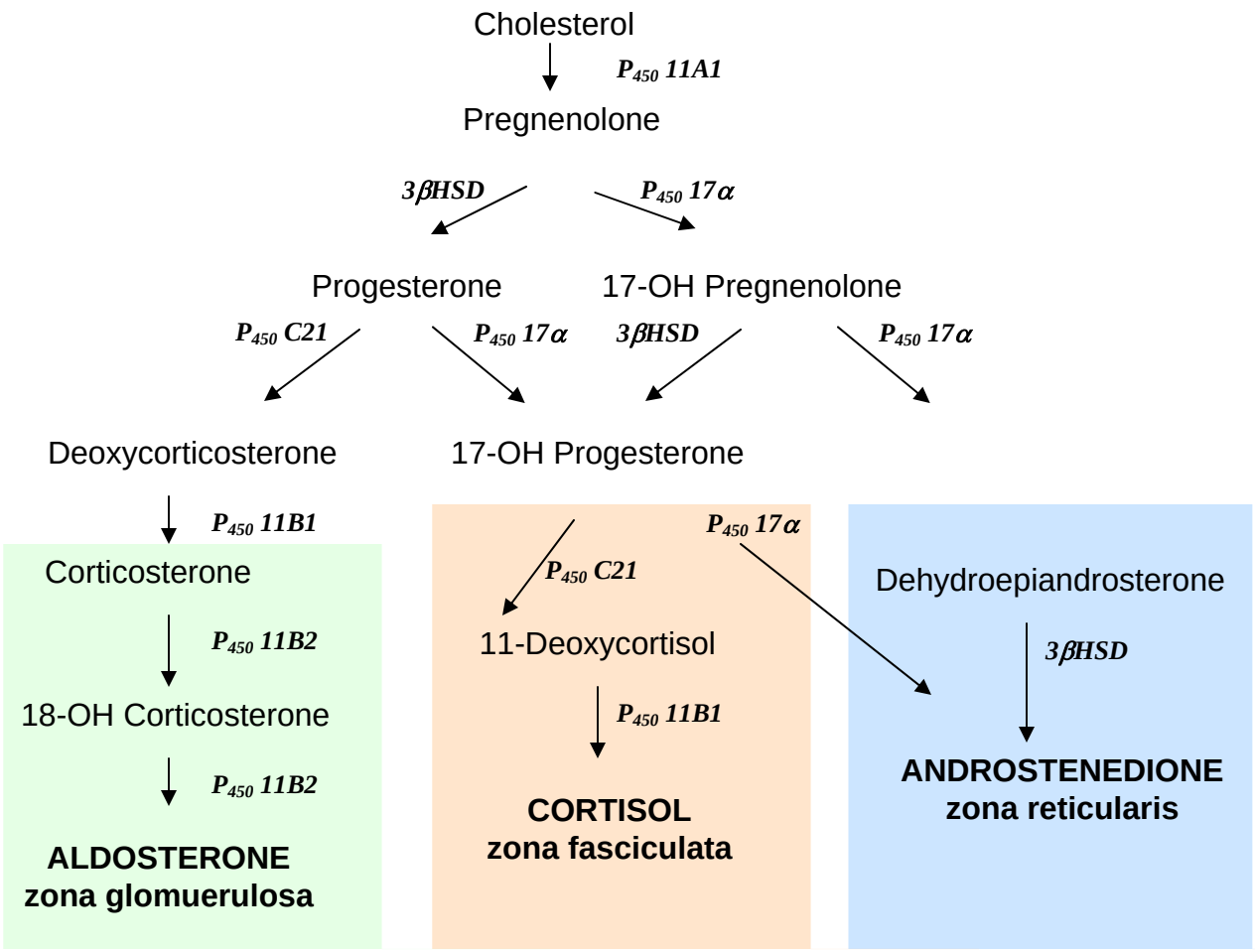

Figure 1-2 Steroid synthesis in the adrenal cortical zones

Each of the adrenal zones synthesizes unique steroid hormones as a result of the expression of distinct genes that are responsible for the terminal conversion of the steroid hormones specific to each adrenal cortical zones. Aldosterone synthase (11β2) and 11β hydroxylase in the zona glomerulosa and fasciculata respectively define zone specific expression of the mineralocorticoid (aldosterone) and glucocorticoid (cortisol in humans or corticosterone in rodents rats) in the two zones.

Between the zones of the zona glomerulosa and the zona fasciculata, there is a unique regenerative zone that has been identified in several organisms including rat, ovine and bovine adrenal glands (Engeland, 1996). This *zona intermedia* possesses none of the terminal enzymes that are required for the synthesis of either the mineralocorticoids (P_{450} 11β hydroxylase) or glucocorticoids (P_{450} aldosterone synthase) and as such occupies an intermediate or undifferentiated state (Fig. 1-3).

The zona intermedia distinguishes the adrenal gland from most other adult mammalian tissues because it has been proposed to function as a zone of differentiation, which gives rise to all the cells of the entire adrenal cortex, thereby acting as a reservoir of adrenal stem cells (Mitani, 1994). The stem cell capability of the zona intermedia is substantiated by experiments that demonstrate bromodeoxyurdine labeled cells originating in the zona intermedia were capable of migrating to either the zona glomerulosa or fasciculata. In addition, a zona intermedia-like cell line was established from adrenal glands isolated from transgenic mice harboring a temperature sensitive mutant of large T antigen simian virus 40 in further support of the stem cell pool that is resident in the normal adrenal tissues (Mitani, 2003; Mukai, 2002).

The stem cell like capacity of the zona intermedia is further supported by evidence that demonstrates the ability of enucleated adrenal glands to regenerate into a fully differentiated and functioning adrenal cortex (Engeland, 1999). The plasticity of the adrenal cortex in this context suggests the presence of inherent processes that would promote and modulate this transition, but little is known about the exact mechanism or cellular components necessary to regulate this phenomenon. The transfer of intercellular signals via gap junctions in concert with hormonal stimuli has been proposed as a mechanism that would impact developmental and other processes in the adrenal cortex (Decker et al., 1978; Green et al., 2001; Shah and Murray, 2001)

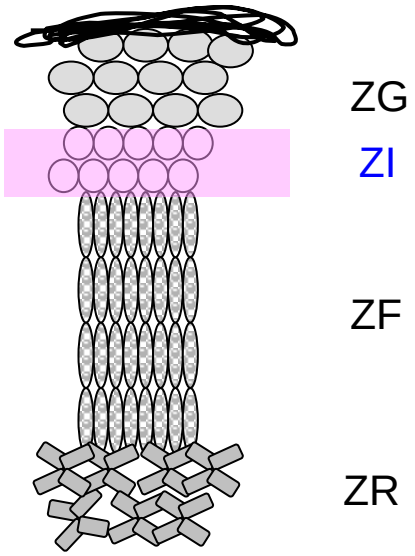

Figure 1-3 Zona Intermedia.

Adrenocortical cells that reside at the transitional zone, the zona intermedia (ZI), (shaded area) between the zona glomerulosa (ZG) and the zona fasciculata (ZF) are postulated to differentiate into all three cells types in the adrenal cortex. These cells express none of the terminial differentiation markers expressed in the other cortical zones, but are thought to acquire different cellular fates as they migrate out of this zone to other areas.

1.2.1. Hormone responsiveness

Adrenal function and homeostasis are governed largely by the hormonal inputs of the hypothalamus-pituitary-adrenal axis. The pituitary gland secretes the adrenocorticotropin peptide hormone (ACTH) in response to corticotropin releasing hormone in the hypothalamus. Once released, ACTH acts differentially on the adrenocortical zones to promote divergent steroidogenic hormones in a zone specific manner (Sewer and Waterman, 2003). For example, the zona glomerulosa is distinguished by a mild responsiveness to ACTH, which can promote the acute secretion of aldosterone during sodium depletion or hyperkalemia. However, chronic exposure to ACTH results in diminished aldosterone synthesis (Ehrhart-Bornstein, 1998). The primary hormonal inputs of the zona glomerulosa are those of the renin-angiotensin signaling pathway which in turn can be modulated by ACTH activity (Quinn and Williams, 1992). The zona fasciculata, in turn, is distinguished by a robust response to ACTH signaling, which results in an array of responses including glandular hypertrophy, steroid secretion and changes in proliferation rates (Liaskos et al., 1998).

Ultimately, the dynamic nature of adrenal gland requires not only the direct hormone-receptor binding of ACTH and other hormones, but also a mechanism that would coordinate glandular signals between the adrenal zones. In accordance with this rationale, the regulation of intercellular signals between the heterogenous adrenal cells in the different adrenal zones may be crucial. Gap junction mediated intercellular communication has been postulated to play an integral role in facilitating the transfer of signals essential for coordinated and synchronized glandular function in adrenal and other endocrine tissues (Meda, 1996a; Meda, 1996b).

However, most studies are based on results obtained from endocrine cells in culture. Cells in culture do not mimic the interactions of the stratified tissues of the adrenal cortex. The study of gap junctions in primary adrenal tissues is facilitates an increased understanding of the nuances of gap junction function as differences in adrenal cellular subtypes may be reflected in their ability to exchange discrete biological signals differentially throughout the cortex.

1.3. Gap Junctions

Gap junctions are present in a variety of tissues. The gap junctions present in the adrenal gland belong to the connexin superfamily of channel forming membrane proteins that comprises at least 20 members in the human genome alone. Indeed in vertebrate tissues, connexins are virtually ubiquitous. The murine genome, for example, contains approximately 19 orthologues to human connexins (Eiberger et al., 2001; Willecke et al., 2002). Although invertebrate phyla such as cnidaria and insects do not express canonical gap junctions per se, they do possess innexins, which are invertebrate analogues to vertebrate connexins. Innexins differ in amino acid sequence typical to connexins yet are functionally identical to the vertebrate connexins (Curtin et al., 1999; Landesman et al., 1999; Phelan and Starich, 2001; Todman et al., 1999). The conservation of gap junction function in such diverse species suggests the importance of gap junction mediated communication in multicellular organisms.

Gap junctions at the cell membrane function as intercellular conduits of small molecules with an upper limit of approximately 1000 Da (Daltons) and are composed of the connexin proteins (Evans and Martin, 2002). The gap junction pore facilitates the passage of several biologically relevant molecules such as glucose, ATP, Ca^{2+}, IP_3, cAMP and other second messengers (Bevans et al., 1998; Cao et al., 1998; Goldberg et al., 2002; Saez et al., 1989a; Saez

et al., 1989b). Their ability to directly transfer second messenger molecules and various ions between cells is the central impetus behind the hypothesis that gap junctions affect numerous cellular processes through the direct intercellular transfer of biologically significant molecules between cells. Therefore, gap junction mediated communication may also be affected by factors, which modulate connexin localization, type and abundance in the plasma membrane.

1.3.1. Connexin Biosynthesis and Organization

Connexin biosynthesis and assembly into functional gap junction pores primarily follows the secretory pathway (Fig. 1-4). Following synthesis on rough endoplasmic reticulum, individual connexin monomers are oligomerized into hexameric connexon hemichannels in the Golgi complex. From the Golgi complex, connexons are transported to the plasma membrane where they dock with the connexons of the apposed cells forming a dodecameric molecule that is the gap junction pore (Musil and Goodenough, 1993; Saffitz et al., 2000; Yeager, 1998; Yeager et al., 1998). Arrays of gap junctions, which consist of several tens to several thousand channels are present at the plasma membrane and are termed gap junction plaques. The turnover and degradation of gap junctions involves both lysosomal and proteasomal pathways. Immature unphosphorylated connexons are postulated to be targeted to lysosomal compartments for degradation, while mature connexins are believed to be processed by the proteasome (Laing et al., 1997; Laird et al., 1991).

The connexin isoforms are all non-glycosylated transmembrane proteins ranging from 25-62 kDa in size (Alexander and Goldberg, 2003; Wang and Rose, 1995). Connexin 43 is the most abundant of all connexin isoforms, and its 3 dimensional structure was the first of all connexins to be solved. A tetramembrane spanning domain, two extracellular loops, one

Gap Junction Biosynthesis and Structure

A.

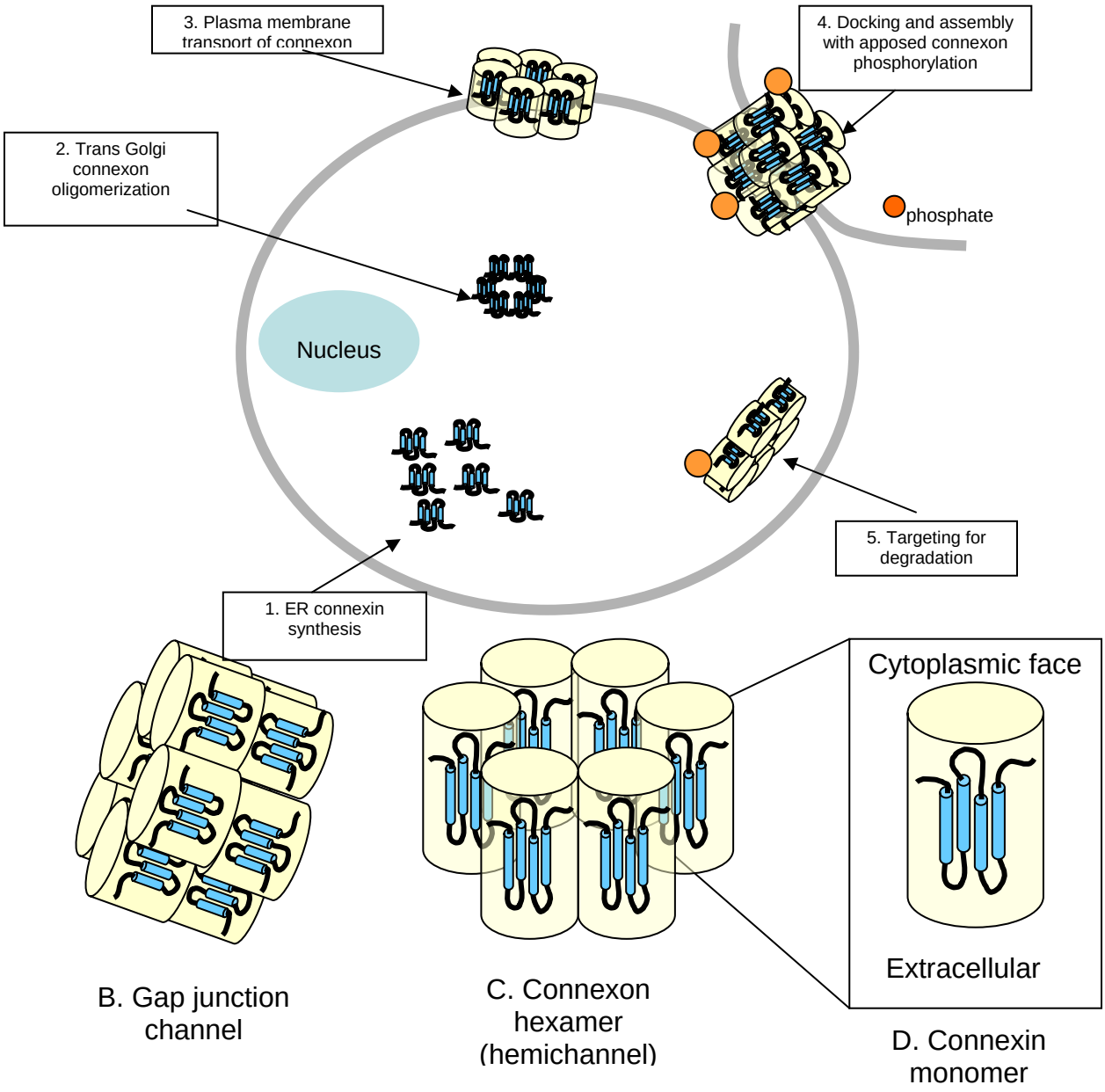

Figure 1-4 Gap junction biosynthesis and structure

The general biosynthetic scheme and structural organization of the connexin molecules, which form the gap junction pore (A). The gap junction channel exists in the membranes of two apposed cells and crosses the 2-4 nm extracellular space ('gap') can be further subdivided into its connexin subunits (B). Each gap junction is composed of two hexameric connexons (hemichannels) (C). Each connexon is formed from 6 connexin monomers. The connexin molecule contains 4 membrane spanning domains, 2 extracellular loops, an intracellular amino and carboxy termini and a single intracellular loop (D).

intracellular loop and intracellular amino and carboxy termini are characteristic of connexin 43 as well as other connexin molecules. Most of the sequence identity between the 20 known human connexin molecules lies in the membrane spanning domains. The second extramembrane domain is postulated to determine actual connexon to connexon interactions and thus alters the combinatorial characteristics of the various gap junctions composed of different connexin molecules (Harris, 2001; White et al., 1994; Yeager et al., 1998). The C-terminus provides the greatest sequence diversity and functional regulation between the different connexin molecules. Ion selectivity and electrophysiological gating of the gap junction channels are influenced strongly by this domain (Veenstra, 1990; Veenstra, 1996).

The gating of gap junction channels is related to specific post-translational modifications of the connexin molecules in addition to the specific connexin subtypes that compose the channels. Changes in phosphorylation, intracellular calcium concentration and pH all affect transjunctional conductances of gap junctions at the cell membrane (Francis et al., 1999; Lampe and Lau, 2000; Lampe et al., 2000). Furthermore, the maintenance and turnover of connexin at the plasma membrane may play critical roles in modulating communication under specific physiological and pathophysiological conditions especially with regard to differences in the respective conductances as well as ion and second messenger selectivity (Musil et al., 2000; VanSlyke and Musil, 2000).

Though formerly considered to be nonselective because of the wide range of molecules and ions it transmits, it is becoming increasingly evident that both the connexin isoform and combinatorial organization of the connexon hemichannels confer molecular selectivity upon various gap junction permeable molecules. Each gap junction has a unique permeability and electrophysiological conductance as a result of the connexin isotype from which it is made.

Primarily, this results from the structural differences within individual connexin molecules (Unger et al., 1999a; Unger et al., 1999b). Gap junctions composed of heteromeric connexons (containing two or more connexin isoforms) exhibit distinct permeabilities to different ions and metabolites than homomeric gap junctions (containing only connexin isoform) (Kavanagh et al., 1994; Veenstra et al., 1995). For example, heteromeric gap junctions of connexin 40 and connexin 43 isoforms exhibit an intermediate electrical and chemical conductance compared to homomeric junctions formed from either isoform alone (He et al., 1999). Therefore, inherent differences occur in the communication potential of the various connexin isoforms. Furthermore, connexons of homomeric and heteromeric composition have been identified *in vivo* and constructed *in vitro* (Diez et al., 1999). Biologically, this is significant as the complement of connexin molecules expressed within different tissues may ultimately affect biological processes in highly tissue specific manners. It is reasonable to propose that alterations in homeostatic regulation through either hormonal stimulus or changes in tissue physiology would impact connexin expression and functional intercellular communication.

1.4. Gap junctions in the adrenal gland

Friend and Gilula were among the first to establish that gap junctions were abundant in adrenal tissues using thin layer and freeze fracture electron micrscopy (Friend and Gilula, 1972). A role for gap junctions in the development of steroidogenic potential was suggested by transmission electron microscopy findings that identified increased cortical gap junctions immediately prior to a surge in corticosteroid levels in mouse, rat and rabbit adrenal glands (Decker, 1981). Subsequent to these findings, however, only a limited number of studies have addressed the physiological significance of gap junctions in the adrenal gland. While gap

junctions are known to be present in most mammalian tissues, and indeed, many other verterbrate and invertebrate tissues, the functional role of connexin expression in the primary endocrine tissues is much less defined. The study of intact tissues may provide informative results that refine the understanding of the diverse biological function of gap junctions in different cell types. In the primary adrenal gland, there is no functional assay demonstrating the relationship between gap junctions and glandular function.

Immunocytochemical studies of gap junctions in the adrenal cortex of bovine, rodent, and ovine tissues have demonstrated a consistent pattern of gap junction distribution and type. The sole gap junction isoforms identified in the adrenal cortex is connexin 43, and its expression is variable througout the cellular subtypes in the three cortical zones. The outer cortical cells of the zona glomerulosa express nearly undetectable levels of the connexin 43 gap junctions in bovine, mouse and guinea pig adrenal glands. The inner cortical zonae fasciculata/reticularis, in contrast, maintain abundant connexin 43 gap junctions (Murray et al., 1995). Connexin 43 distribution in the adrenal cortex is also coincident with physiological differences in proliferation rates and steroid production throughout the three adrenocortical zones as well. For example, the secretion of glucocorticoids and androgens in the connexin 43 abundant zonae fasciculata and reticularis respectively contrasts the mineralocorticoid secreting parenchymal cells in the zona glomerulosa. In the human adrenal gland, the gap junction distribution has not been previously described though hormonal secretions and organization are similar in adult humans to that observed in rodents (James, 1992). As there may be clinical relevance to understanding gap junction mediated communication in adrenal disorders, an exploration of gap junctions in human adrenal tissues was also warranted. The primary objective of these investigations detailed herein is to

gain insight into how communication through gap junctions might impact function and potentially contribute to the development of disorders which disrupt adrenal physiology.

In terms of adrenal physiology, hormonal responsiveness of endocrine tissues is postulated to be due to not only receptor-ligand binding but also to the transmittal of hormonal signals through gap junctions. Direct exchange of signals through gap junctions may play a substantial role in mediating hormonally derived signals in exocrine and endocrine tissues (Meda, 1996b; Meda et al., 1991; Stock, et al. 1998). For example, adrenal cultures that express gap junctions exhibit a dependence on gap junctions for both steroid secretion and proliferation (Munari-Silem et al., 1995). In these studies, ACTH stimulated cultures demonstrated increased steroid production with a concomitant decrease in rates of proliferation. The stimulatory effect of ACTH on adrenal cell steroidogenesis was attenuated subsequent to either pharmacological inhibition of gap junctions or by transfection of an anti-sense gap junction construct (Shah and Murray, 2001). The data demonstrating a responsiveness of cultured adrenal cells to hormonal treatment support the presence of an intercellular communication mechanism that is gap junction mediated.

Functional implications in the adrenal cortex

The glandular organization of the adrenal cortex provides an informative model system in which to examine gap junction mediated communication and its potential role in tissue homeostasis and function. The heterogeneous cell types of the adrenal cortex exist in a zonal framework and, as a result, provide a means to evaluate the relationship between adrenal physiology and cell-cell communication in different subpopulations of cells the defined adrenal zones. This thesis explores how the distribution of gap junctions and their resultant communication profile are manifested in the tissues of adrenal cortex itself. Furthermore, the

impact of disturbing adrenocortical physiology through the disruption of the hypothalamus-pituitary adrenal axis and adrenal gap junction will be evaluated to better define the functional role of gap junction mediated communication in adrenal cortical function.

Cellular morphology, proliferation and steroidogenic capacity in the specialized adrenocortical zones reflect its physiological and functional stratification (Bland, 2003). Therefore, the coordinate action of the adrenal cortex must not only be governed by strict control of gene expression, but also by the synchronicity of the different cellular subtypes within the gland. Gap junction mediated intercellular communication has been postulated to play an integral role in facilitating the transmission of signals essential to adrenal and other endocrine-neuroendocrine organ function (Meda et al., 1993; Serre-Beinier et al., 2002). For example, selective stimulation of granulosa cells in adrenal/granulosa cell co-cultures with follicular stimulating hormone (FSH) resulted in the activation of cAMP dependent protein kinase A and steroid production in the adjacent adrenal cells indicating direct intercellular communication of the cAMP second messenger (Murray and Fletcher, 1984). This study, while informative, does not reflect the dynamics in the heterogeneous cells of the adrenal cortex. It is necessary to deepen our understanding of the gap junction profile within the adrenal gland to elucidate the role gap junctions play in the diverse landscape of the adrenal cortex. First, it is necessary to establish the fundamental characteristics and distribution of the connexin proteins in the context of the adrenal gland itself. Subsequently, an exploration of the potential functional role of the resident adrenocortical gap junction can be assessed.

Physiologically, the potential requirement for gap junction communication in the adrenal gland as well as in a variety of tissues and organ systems seems imperative, as the nearly ubiquitous presence of the connexin proteins would suggest. Loss of connexin in multiple cell

types through either mutation or impaired regulation results in a number of maladies affecting diverse organs and tissues. Mutational analysis of the connexin genes has provided insight into the potential contribution of gap junctions to a variety of functions in diverse tissues. One of the most prominent examples is that of the X-linked Charcot Marie Tooth Disorder (CMTX), which is manifested as various peripheral neuropathies as a result of multiple mutations in the connexin 32 gene. A genetic analysis of the connexin 32 gene in cohorts of individual afflicted with the disorder revealed mutations at several positions in the connexin 32 gene that result in variations of both severity and time of onset of symptoms (Yum et al., 2002). Connexin 26 mutations in cochlear hair cells are associated with nonsyndromic deafness (Denoyelle et al., 1998; Zelante et al., 1997), while connexin 50 and 46 mutations in ocular lens cells are related to the development of cataracts (White et al., 1998). Connexin 43 and 40 mutations in cardiomyocytes have been correlated to cardiac arrhythmias and faulty electrical conduction in the heart are also among a growing number of disorders in which mutations of the gap junction genes is linked to disease development and presentation (Severs, 1999; Severs, 2000) (see Table 1).

Transgenic mouse studies further support the importance of gap junction mediated communication in proper tissue function and development. Genetic deletion of the connexin 43 gene, which is enriched in the adrenal gland, cardiomyocytes and several other tissues, results in postnatal lethality due to right ventricular malformations (Meyer, et al., 1997; Plum, et al., 2000; Reaume et al., 1995; Roscoe et al., 2001). There is also growing evidence that expression of specific connexin isoforms is required in diverse tissues. For example, while perinatal lethality was rescued in connexin 43 deficient mouse strains in which the connexin 32 gene was “knocked in,” homozygous male pups were infertile due to a germ line deficiency (Plum et al., 2000). The transgenic mouse studies and the mutational analysis of connexin gene support a fundamental

role for gap junctions that is critical for homeostatic regulation, development and function that is highly tissue specific.

It is the potential tissue specific function of gap junction that is relevant to their role within the landscape of the adrenal cortex. For example, gap junction expression in adrenal cells *in vivo* and *in vitro* is inversely related to proliferation. The mitotic index of zona fasciculata/reticularis is appreciably less than that seen in the zona glomerulosa (Murray et al., 1995). *In vitro* a similar anti-proliferative response has been observed in cultured adrenal cells and several other endocrine tissues. Adrenal and thyroid cells transfected with gap junction constructs typically demonstrate a reduced rate of proliferation (Guerrier et al., 1995; Murray and Shah, 1998). The anti-proliferative function of gap junctions in the inner cortex is complemented by findings that established high levels of apoptosis in the zona reticularis of rat adrenal cortex following removal of endogenous ACTH signals through pituitary resection (hypophysectomy). The high level of apotosis in the zona reticularis was also blocked by the administration of exogenous ACTH (Carsia, et al., 1998; Carsia, et al., 1996). While gap junction expression was not investigated in these studies of apoptosis, it is particularly relevant here as the demonstrated to transmit apoptotic signals involved in both normal tissue homeostasis and in the. zona reticularis, as stated previously, is abundant in gap junctions. As gap junctions have been inhibition of neoplastic transformation, their role in normal tissue physiology and may extend to maintenance of overall tissue integrity necessary for tissue specific function (DeoCampo et al., 2000).

Table 1 Association between Connexins and Disease

Connexin (Cx)	Tissue Expression	Mouse Knockout	Human Disease
Cx25	ND		
Cx26	liver, brain, cochlear hair cells	Embryonic lethal	Nonsyndromic deafness
Cx30	skin		Skin disease
Cx30.3	skin		Skin disease
Cx31	skin		Skin disease
Cx31.1	skin stroma		
Cx31.3	ND		
Cx31.9	vascular smooth muscle		
Cx32	liver, Schwann cells	Hepatocarcinoma	Peripheral neuropathy
Cx33	testis		
Cx36	retina, brain		
Cx37	lung	Female infertility	Myocardial infarct
Cx40	heart		Cardiac arrhythmia

Table 1 cont'd

Cx43	Heart, lens, brain, adrenal cortex	Abnormal heart development, perinatal lethality	Oculodentodigital dysplasia, cardiac arrhythmia
Cx45	Heart	Defective vascular development	
Cx46	Ocular lens	Cataracts	Cataracts
Cx50	Ocular lens	Cataracts & microphthalmia	
Cx57	Skin, Heart, kidney		
Cx59	ND		
Cx62	ND		

*** Modified from Alexander and Goldberg 2003*
ND = not determined

Several questions remain with regard to how intercellular signals are manifested in stratified tissues with variations in gap junction distribution. Results from tissue culture experiments fail to explain how relatively uniform signals from adrenotropic hormones such as ACTH are disseminated through the heterogeneous population of cells of the adrenal cortex. In this thesis, the hypothesis that the differential distribution of gap junctions in the adrenal gland reflects the glandular trophic state will be tested. The trophic state of the adrenal gland will be altered by hypophysectomy and its effect on the glandular architecture and gap junction expression will be demonstrated. Gap junction expression in normal and neoplastic tissue will be investigated to explore the potential relationship between gap junctions and adrenal pathology. Furthermore the functional communication of native adrenal gap junctions will be evaluated.

2. CHAPTER 2 METHODS

2.1. Immunocytochemistry

Connexin antibody description and immunostaining

The rabbit polyclonal gap junction specific antibodies used to evaluate tumor specimens (IgG) were gifts of the late Drs. Norton B. Gilula (Scripps Research Institute LaJolla, CA) and Nalin Kumar (University of Illinois). The antibodies were prepared and characterized against synthetic peptides corresponding to cytoplasmic domains between transmembrane domains 2 and 3 or the carboxyl end of the connexin molecules: connexin 43 (residues 370-380), connexin 32 (residues (252-280) and connexin 26 (residues 112-115). The antibody characterization and preparations have been previously published (Risek et al., 1994).

Connexin antibody immunostaining

Cryosections of adrenal specimens were embedded in cryoprotective O.C.T. medium and cut on a microtome. Cut adrenal specimens were allowed to come to room temperature. The slides were washed in PBS for 3-5 min. to remove the embedding medium. Specimens were demarcated with a pap pen and inbubated in a 3% BSA, 3% goat serum blocking solution in PBS for 45 min.-1 hr. at 37°C. After blocking, the adrenal specimens were incubated with anti-connexin rabbit polyclonal 43, 26, or 32 diluted in blocking solution for 1hr at 37°C or at room temperature overnight. Connexin antibodies were obtained from Zymed Laboratories (San Francisco, CA) or those received as a gift from Dr. Norton Gilula and were found to produce identical staining patterns in the adrenal specimens studied. The specimens were washed three times in PBS, incubated in secondary (goat anti-rabbit) antibody conjugated to either a Cy3 or Alexa 594 fluorophore (Molecular Probes, Eugene OR) for 1 hr. and washed 3 times in PBS.

Specimens were treated with Hoescht dye to visualize their cell nuclei, mounted in Fluoromont G antiquenching reagent and placed under a glass coverslip for microscopic analysis.

Cytochrome P_{450} aldosterone synthase antibody

The rabbit anti-rat cythochrome P_{450} aldosterone synthase (P_{450} aldo) and 11β hydroxylase antibodies were generous gifts from Dr. Gomez-Sanchez (University of Missouri, Columbia, MO) (Wotus et al., 1998). To immunolabel the adrenal specimen with P_{450} aldo, frozen tissues slices were treated with a blocking solution (3% goat serum and 3% BSA and 0.1% Triton X 100 for 45 min.-1hr at room temperature. The adrenal specimens were then incubated with the P_{450} aldo antibody dissolved in the blocking solution at room temperature overnight. Following the incubation, the glands were washed in PBS containing 0.1% Triton X 100 and treated with a goat anti-rabbit Alexa 488 or Alexa 594 conjugated secondary antibody for approximately 1hr. at room temperature. The specimens were then washed in PBS, treated with the Hoescht anti-nuclear dye and mounted in Fluoromont-G antiquench solution.

2.2. Human adrenal specimens

To analyze gap junctions in aberrantly proliferating cell populations, human adrenal tumor samples were evaluated from donor individuals undergoing tumor resection. Normal adrenal glands were donated by individuals undergoing nephrectomy. Nine tumor and three normal specimens were evaluated for gap junction distribution. The adrenal tissues investigated were classified by Stephan Bornstein, M. D. (Heinrich-Heine University, Germany) as follows: three were characterized as adenomas with autonomous cortisol secretion, six were carcinomas (malignant tumors) associated with the production of various steroids and three were normal

tissues. The cortical origin of the tumors was confirmed by immunohistochemical staining against cytokertatin, vimentin, synatophysin and D11 protein (Marx, et al., 1996). The malignant state of the tumors was diagnosed or excluded according to the published criteria (Hough, et al., 1979; van Slooten, et al., 1985; Weiss, et al., 1984). Briefly, adrenal malignancy was characterized by increased mitotic rate, nuclear grading, atypical mitosis, clear cells comprising 25% or less of the tumor mass, diffuse patternless sheets of cells, necrosis, invasion of venous structures, sinusoidal or capsule of the tumor and the presence of metastasis. The six carcinomas did not express the MHC (major histocompatibility complex II), a previously determined marker found to be absent in adrenal tumors (Marx, et al. 1996).

2.3. Image analysis of adrenal tumors

Microfluorometric image analysis of adrenal gap junction number and size were determined using the Optimas Image analysis program (Media Cybernetics, Silver Spring, MD). Zona reticularis cells close to the medullary border were selected in order to avoid the possibility of mixing zona fasciculata and zona reticularis cells. Cells in the zona fasciculata near the border of the zona glomerulosa were selected for analysis. As most carcinomas arise from the zona fasciculata in adrenal tissue, this adrenal zone was used for comparison of gap junctions in the tissue specimens. Adrenal gap junctions were quantitated by averaging 20 or more representative areas from two adrenal slices from each patient specimen. Cell numbers were obtained by counting Hoescht dye stained cell nuclei. Images analysis was performed using a Nikon Microphot FXA fluorescence phase microscopes interfaced to the Optimas Image Analysis program run on a Gateway computer (Gateway 2000 Inc., North Sioux City, SD) or by manual counting of captured cell images. Statistical analysis between the means of the gap

junction numbers was calculated using the Student's *t* test. Data were expressed as the mean value ± SEM to a stringency of $p < \text{or} = 0.05$.

2.4. Hypophysectomy

To elucidate the relationship between gap junctions and adrenal gland homeostasis, we evaluated adrenal glands from hypophysectomized animals. Six week old C57/Bl6 female were hypophysectomized via a parapharangeal method at the Charles River Laboratories (Wilmington, MA, USA). Following hypophysectomy animals were housed in the University of Pittsburgh Animal facility and fed a diet of standard mouse chow (Purina) and given water supplemented with 5% sucrose ad libitum. Several hypophysectomized animals received exogenous ACTH (adrenocortocotropin hormone Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection at a concentration of 1U/gm body mass in a saline solution 1-3 hours before harvesting the adrenal glands.

To harvest the adrenal glands, animals were first anesthetized with halothane (Halocarbon Laboratory, River Edge, NJ) via inhalation and sacrificed via cardiac puncture. Freshly excised adrenal glands were embedded in cryoprotective (OTC) embedding medium, flash frozen in liquid nitrogen and stored at –80°C. Adrenal glands were sectioned using a crysostat at 5-8 μm for immunological or histochemical analyses. Adrenal specimens were collected at 7, 14, 33, 64 and 110 days post-hypophysectomy. Quantitative analysis was performed on cohorts of 4 animals for each experimental control and hypophysectomized animals with the exogenous ACTH injection in which 2 hypophysectomized animals were evaluated.

2.5. Transgenic mice

Connexin 43 transgenic mice that were deficient for the connexin 43 gene (Gja1) and neonatal adrenal glands specimens characterized here were generous gifts from Dr. Ceclia Lo (University of Pennsylvania, Philadelphia, PA). The presence or absence of the connexin 43 protein was determined by Western blot analysis using an anti-connexin 43 polyclonal antibody (described under Immunohistochemistry).

Stereological Imaging Methods

Gap junction size and number were determined using computer assisted image analysis of the immunolabled adrenal glands and expressed as gap junction/cell or gap junction/area. Stastical comparison of mean values ($_{SEM}$) was performed using a one-way analysis of variance and the Duncan's Multiple Comparison Procedure. Statistical significance was measured by employing Student's *t* test.

2.6. Cell culture

The SW-13 human adrenal carcinoma cell line was obtained from American Tissue Type Cell Culture (ATCC, Manassas, VA) and maintained in L-15 nutrient medium (Grand Island Biological, Grand Island, NY) containing fetal calf serum (10%), cortisone (10 mg/ml), insulin

(0.02 mg/ml, streptomycin (0.1 mg/ml), fungizone (0.01 mg/ml) buffered with L-arginine to pH 7.2. Cultures were maintained at 95% humidity and 5% CO_2 at 37ºC.

H295 cells

The H295 human adrenal carcinoma cell line obtained from (ATCC) (Gazdar) cells were maintained in DMEM-F-12 (Gibco-BRL) medium supplemented with 1% ITS, 1% penicillin/streptomycin and 2.5% NuSerum (Calbiochem, La Jolla, CA). Cells were maintained in a 95% humidity and 5% carbon dioxide at 37°C.

2.7. Gap junction mediated communication assays

2.7.1. Scrape loading assay

Gap junction mediated cell-cell communication was assessed with an established procedure as previously described (el-Fouly et al., 1989). Cells from the SW-13 adrenal tumor cell line were cultured on coverslips in 30 mm culture dishes until they reached ~80-90% confluency in L15 medium at 95% humidity 5% CO_2. The cultures were then washed in EBSS (Earle's Buffered Saline Solution) pH 7.4 to remove residual medium. The cultures were then placed in an EBSS solution containing 0.5% Lucifer Yellow (EBSS/LY) (Molecular Probes, Eugene OR USA). The cultures were bathed in the EBSS/LY solution and scored with a Pasteur pipet tip to provide initial entry of the Lucifer Yellow dye into the cells. The EBSS/LY solution was removed after 2 minutes and the cultures were washed briefly in EBSS and fixed in 4% paraformaldehyde. Cell nuclei were stained with Hoescht nuclear dye, and the coverslips were mounted in Fluoromont G anti-quench solution in preparation for microscopic analysis.

2.7.2. Whole gland communication assay

Intercellular communication in primary mouse or primate (rhesus monkey, Maccaca mulatta) adrenal tissues was assessed by incubating the tissues in a cell communication bath, which consisted of was 0.5% Lucifer Yellow CH (443 Daltons) (Molecular Probes, Eugene, OR) dissolved in PBS pH 7.4 (phosphate buffered saline) and/or 0.5% dialyzed rhodamine dextran (10,000 Daltons, Sigma-Aldrich, St. Louis, MO). The gap junction specific inhibitor, oleamide dissolved in 65% ethanol (Sigma-Aldrich, St. Louis, MO) was added to the dye communication bath to a final concentration of 100-200 μM to block gap junction mediated communication in the intact adrenal tissues. The assay was performed as follows: adrenal glands were surgically removed with care to remove excess subcutaneous fat and tissue from the glands. Whole or severed adrenal glands were incubated in the communication bath for 10 min. at 37°C. Following the incubation in the communication bath, the glands were washed in PBS and fixed for 1-2 hrs in 4% parformaldehyde at room temperature, embedded in O. T. C. cryoprotective medium, flash frozen in liquid nitrogen and stored at –80°C prior to analysis.

3. CHAPTER 3 GAP JUNCTION MEDIATED COMMUNICATION IN THE INTACT ADRENAL GLAND

The presence of functional gap junction driven communication within tissues expressing gap junctions is a common presumption that often remains undetermined empirically. Tantamount to the expression of gap junctions is the verification that functional gap junction mediated communication exists within tissues that express connexins. In the adrenal cortex, for example, the abundance of connexin 43 expression in the zonae fasciculata and reticularis suggests active and abundant communication within these regions. However, mere visualization of gap junctions in a tissue is not a conclusive determinant of communication networks between the heterologous populations of cells within a tissue. Gap junction activity is known to vary depending on particular physiological requirements in tissues. Networks of astrocytes or neurons in the brain, for example, may occupy similar regions but restrict intercellular communication to subpopulations of cells that are in their anatomical proximity (Hagendorff et al., 2001; Krutovskikh et al., 2002). Furthermore, there is evidence that gap junctions present in tissues or in cultured cells are inactive as a result of post-translational modifications and alterations of physiological state (Duthe et al., 2000; Li and Nagy, 2000; Rivedal et al., 1994; Wang and Rose, 1995).

Microinjection has proven to be a viable assessment of gap junction mediated intercellular communication in endocrine cells by the transfer of gap junction permeable dyes to neighboring cells (Halban et al., 1982; Morand et al., 1996). However, gap junction mediated communication has never been demonstrated in the intact adrenal gland. Understanding the communication patterns in the intact tissue would overcome some of the limitations inherent in cultured cells. For example, primary adrenal cultures lack the heterogeneity of cells types and

zonation found in the adrenal cortex. Zona glomerulosa and zona fasciculata primary cocultures results in the loss of the zona glomerulosa phenotype (Mazzocchi, et al., 1986). Adrenal cell monolayers are also comparatively uniform in their connexin expression in contrast to the heterogeneity of connexin expression in the individual zones of the intact gland. Therefore, a novel assay that identified and characterized functional communication in the intact gland was necessary.

The introduction of gap junction permeable dyes into the adrenal cortex of the intact gland via microinjection is both difficult and impractical as the number of cells that would be required for informative gland-wide analysis of dye communication patterns would be enormous (Meda, 2000). Therefore, to determine if the abundance of connexin expression in the adrenal cortex is translated into functional gap junction mediated communication, a whole gland dye communication approach was developed (Fig. 3-1). It was the goal of these experiments to: 1) verify that communication in the adrenal cortex is mediated by endogenous connexin 43 identified through immunocytochemistry, 2) determine if the resident gap junctions were indeed functional in living tissues and 3) eliminate the possibility of the presence of other connexin isoforms in the outer cortex (zona glomerulosa) of the intact gland through a functional assay.

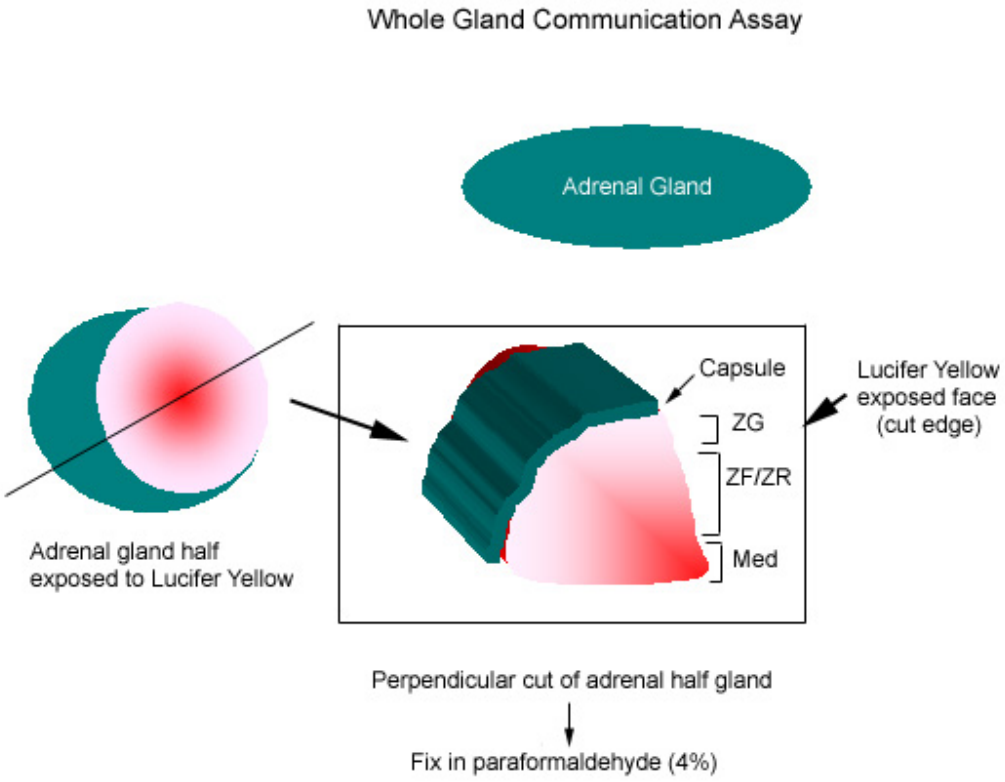

Figure 3-1 Protocol for whole gland cell-cell communication assay

An alternative protocol to the scrape loading assay in cultured monolayers of cells was employed to evaluate gap junction mediated communication in primary adrenal tissues. Freshly excised adrenal glands were halved and placed in the communication bath containing the gap junction permeant dye Lucifer yellow. After the incubation, glands were fixed in paraformaldehyde and flash frozen in liquid nitrogen for microscopic analysis.

Results

The SW-13 human adrenal tumor cell line was analyzed for the presence of connexin 43 gap junctions and gap junction mediated communication. An immunocytochemical analysis of the SW-13 cells revealed abundant connexin 43 gap junctions that were characteristized their punctate fluorescence at points of cell-cell apposition. Cell-cell communication between SW-13 adrenal cells was demonstrated by a scrape loading assay (Fig. 3-2). Dye communication of the gap junction permeable dye Lucfier Yellow (443 Da) confirmed functional gap junction mediated communication between connexin 43 positive adrenal cells in culture. The SW-13 cells efficiently transferred Lucifer Yellow omnidirectionally to cells having direct cell-cell contacts from injured cells at the scored edge to neighboring cells in the monolayer (denoted cut edge).

Gap junction expression and cell-cell communication profiles were characterized in the intact mouse adrenal cortex by immunocytochemical analysis and a communication assay modified for intact tissues (Fig. 3-1). At low magnification in a nicked adrenal gland, the outer cortical cells were virtually devoid of gap junction staining as demonstrated by the absence of connexin 43 in the outer cortical regions (Fig. 3-3). The near absence of connexin 43 staining in the outer cortical regions is contrasted by the high level of staining within the inner 2/3 of the adrenal cortex. The whole gland dye communication assays revealed a pattern of Lucifer Yellow dye communication that was parallel to connexin 43 staining in the inner two-thirds of the adrenal cortex (Fig. 3-3). The outer cortical regions were typified by the absence of Lucifer Yellow dye communication, while the inner cortical zones were communication competent. A distinct zonal character of communication and connexin 43 expression was observed at a higher magnification of the tissue. The parenchymal cells of the zona glomerulosa near the adrenal

capsule expressed no connexin 43 and were incapable of dye communication (Fig. 3-4). This is consistent with connexin 43 expression in this zone as the fibroblasts in the outer connective tissue capsule and its trabeculae expressed the connexin 43 protein and were also capable of dye transfer. In the rare instance when Lucifer Yellow was observed within zona glomerulosa, it was restricted to isolated cells and not between two or more cells.

To confirm that the Lucifer Yellow dye transfer observed was indeed gap junction mediated, severed adrenal glands were placed in the communication bath and viewed from the cut edge (Fig.3-5). All cells at the cut edge of the tissue contain the Lucifer Yellow dye. However, cells in the zona glomerulosa were unable to transfer dye past the cut edge. The cells of the outer cortical zona glomerulosa were deficient in their ability to transport Lucifer Yellow dye to cells immediately proximal to the site of cell injury demonstrated by the brightly staining cells at one or two layers in depth within the zona glomerulosa.

The cells from the two inner adrenal zones were able to transfer Lucifer Yellow dye. The cells of the zonae fasciculata/reticularis were able to transfer dye to multiple cell layer as shown in (Fig.3-5). Dye transport within communication competent zonae fasciculata/reticularis cells was not uniform. Cells bordering the zona glomerulosa transported less dye than cells deeper in the inner cortical regions. This communication pattern is also coincident with the immunostaining pattern of connexin 43, which is most abundant within the deeper zonae fasciculata and reticularis. Furthermore, a zone specific dye communication pattern across the adrenal cortex supports an inherent difference in the requirement for communication between physiologically different cells of glucorticoied producing zona fasciculata versus that of the mineralocorticoid producing cells of the zona glomerulosa.

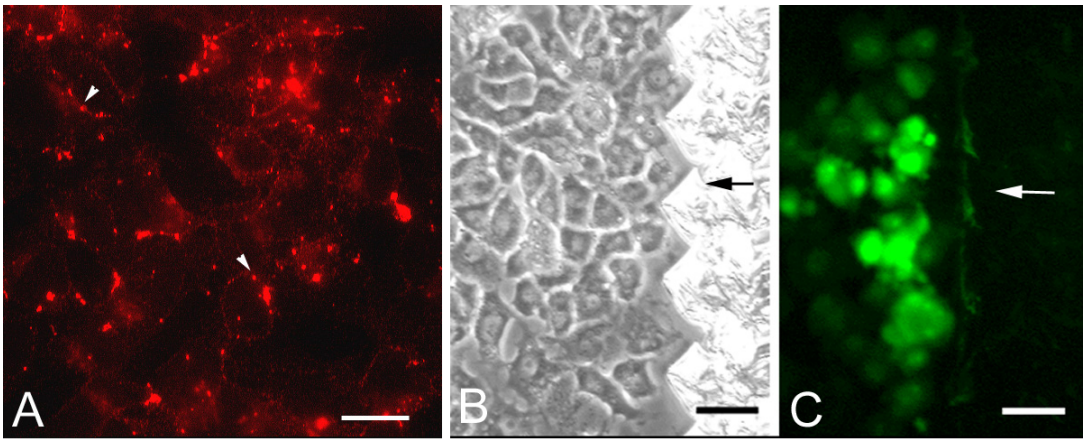

Figure 3-2 Connexin 43 gap junction expression and dye communication assay in SW-13 adrenal tumor cell line

Indirect immunofluorescence of SW-13 adrenal cell line with anti-connexin 43 antibody (A). Adrenal cells expressing characteristic punctate flurorescence of gap junctions (arrowheads) at the cell surface. Phase contrast image showing confluence and morphology of adrenal cell monolayer (B). Fluorescent image (green) of scrape loaded cells demonstrating dye transfer of the gap junction permeant Lucifer Yellow (443 Da). Cells communicate the dye extensively to neighboring cells (C). Arrows (B & C) indicate initial point of dye entry from injured cells at the cut edge. Bar in A = 20μm. Bar in B & C = 50μm.

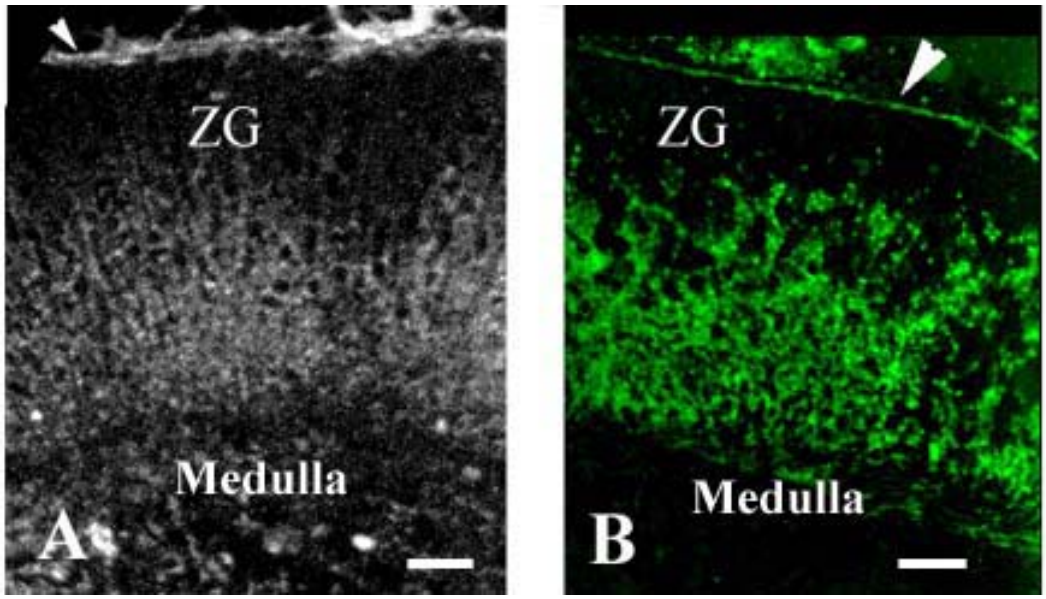

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Figure 3-3 Immunolocalization of connexin 43 and cell-cell communication in the intact adrenal cortex.

Connexin 43 was localized using a connexin 43 specific antibody to the inner cortical regions of the adrenal cortex. The diffuse pattern of fluorescence indicates the abundant connexin 43 typical at low magnification (A). In a separate adrenal specimen, abundant dye transfer is evident in the nicked whole adrenal gland incubated in the presence of the gap junction permeant Lucifer yellow (green) (B) and parallels connexin 43 expressions. Arrowheads = adrenal capsule. Bars = 50 μm.

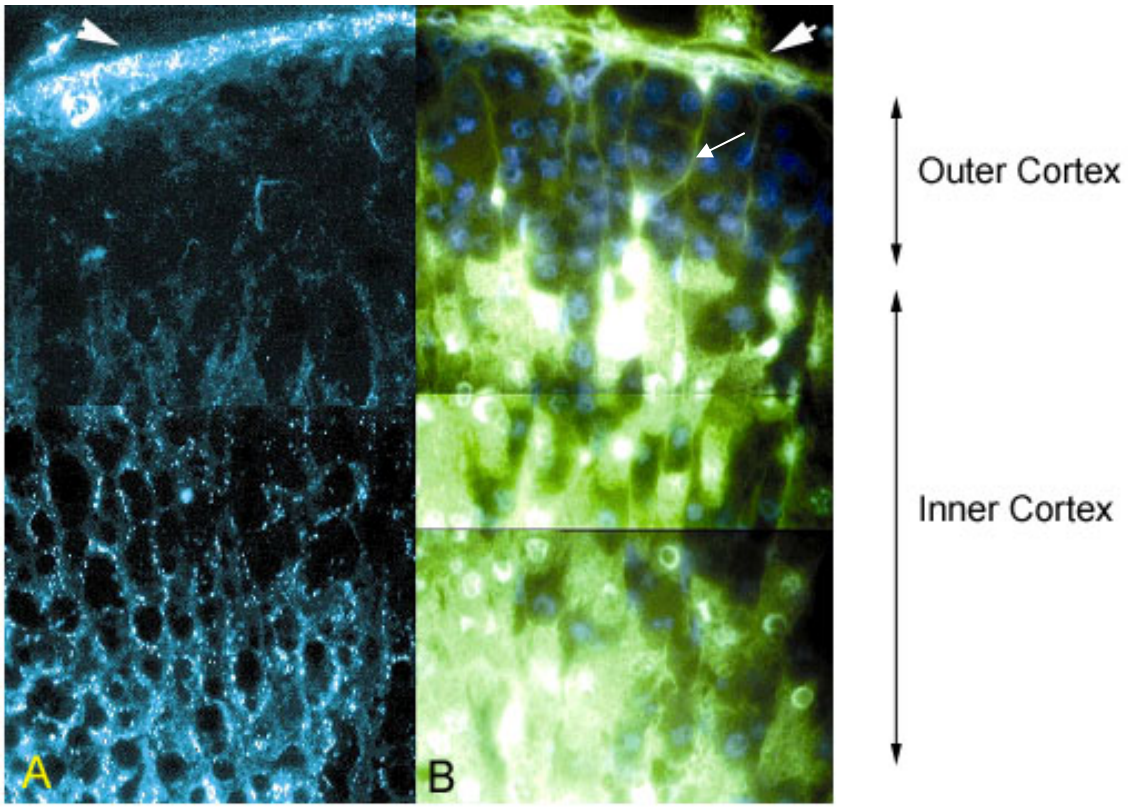

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Figure 3-4 Connexin 43 immunocytochemistry and gap junction mediated intercellular communication in the outer and inner cortex.

Connexin 43 gap junctions are abundant within the inner cortical regions and the adrenal capsule and as demonstrated by their punctate fluorescence using a connexin 43 specific antibody (A). The outer cortex beneath the adrenal capsule has few detectable gap junctions. Lucifer Yellow dye communication (cell-cell coupling) indicating gap junction driven intercellular communication is extensive in the cortex parallels regions of gap junction abundance (B). The connective tissue extensions that outline the zona glomerulosa parenchymal cells (arrow) are communication competent but do not transfer dye to the communication incompetent zona glomerulosa.

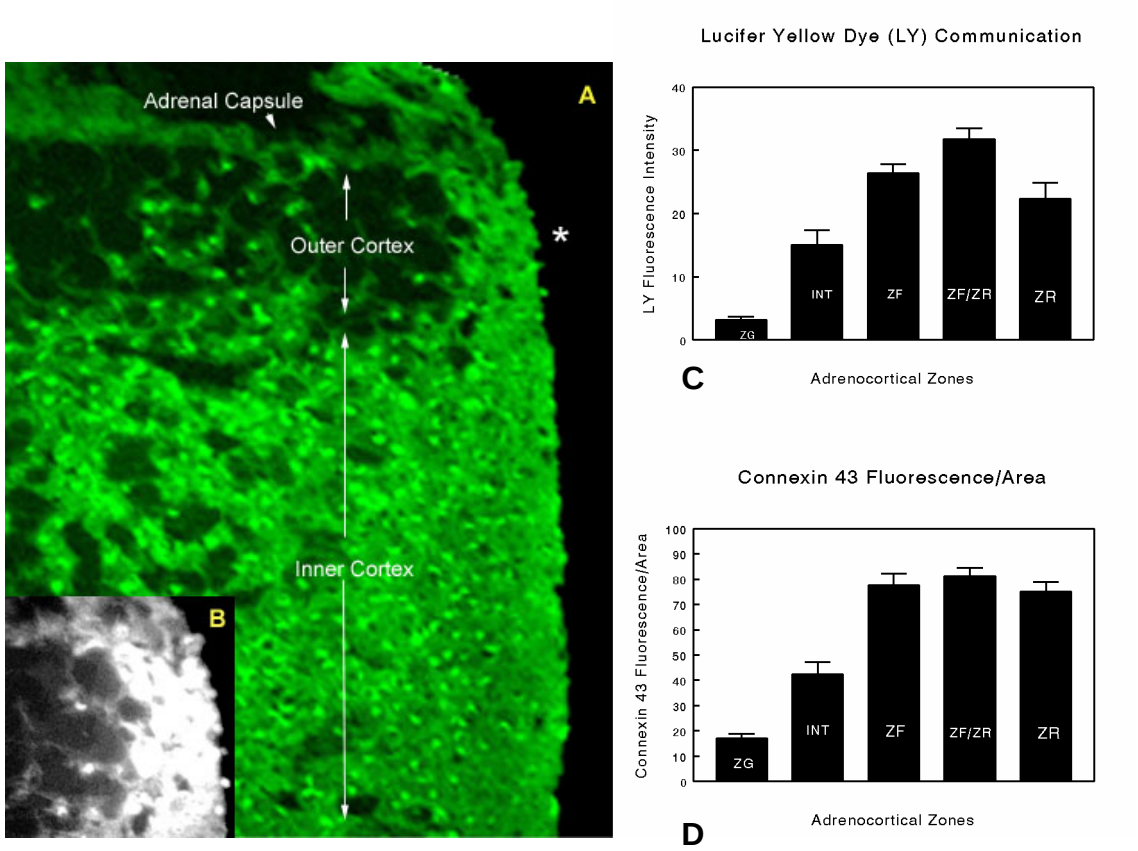

Figure 3-5 Cell-cell communication at the cut edge

The whole gland dye communication assay was evaluated from the site of entry (cut edge) of the gap junction permeable dye Lucifer Yellow. Transmittal of the Lucifer Yellow dye was differential throughout the adrenal cortex. In the subcapsular zona glomerulosa (ZG) , cells outside of the area of the injury are incapable of transferring Lucifer Yellow dye (A, B). In contrast, deeper cortical cells transfer Lucifer Yellow dye through multiple cell layers (A). An enlargement of ZG illustrates the inability of cells in this adrenal zone to transfer dye beyond the cut edge. Inset (B). Fluorometric measurements of gap junctions (C) and dye communication (D) showed the direct relationship between gap junction abundance and the degree of intercellular communication. Asterisk in (A) = cut edge.

Quantitative analyses confirmed the qualitative microscopic observations. When fluorescence intensity values of dye communication are compared to that of the immunocytochemical staining for connexin 43, the relationship between communication and connexin abundance was confirmed (Fig. 3-5). There is also a direct correlation between the amount of connexin 43 expression and dye communication within the murine adrenal cortex. Dye communication is highest in the zona fasciculata as is connexin 43 expression. These analyses also allowed us to eliminate the possibility of the existence of an unidentified connexin isoform within the cortex. To date, only connexin 43 has been identified in the adrenal cortex via immunocytochemical analysis (Murray et al., 1995). The dye communication experiments demonstrate that dye communication is consistent with the presence of the connexin 43 isoform. If an unidentified connexin were present in the adrenal gland zona glomerulosa, it is likely that it would facilitate Lucifer Yellow transport. However, while the presence of heteromeric connexin between the zona glomerulosa and the zonae fasciculata/reticularis is still possible, it is unlikely that the zona glomerulosa maintains gap junction mediated communication since the paucity of immunohistochemical and dye communication data does not support the presence of gap junctions in the zona glomerulosa. Alternatively, an unidentified connexin isoform in the zona glomerulosa that is impermeable Lucifer Yellow is still possible and would indicate a communication network in the outer cortex that is distinct from the inner cortical zones.

Gap junction mediated communication was also confirmed in control experiments in which the gap junction inhibitor oleamide was used. Oleamide is a naturally occurring fatty acid that selectively inhibits gap junction mediated communication. It has been employed to disrupt the propagation of gap junction specific signals involved in homeostatic and developmental processes (Boger et al., 1998; Guan et al., 1997; Huang et al., 1998a). Whole gland

communication experiments done in the presence of oleamide revealed no significant transfer of Lucifer Yellow in any of the three cortical zones independent of connexin abundance (Fig. 3-6). Typically, gap junction mediated intercellular communication observed in oleamide treated adrenal specimens was significantly reduced and occasionally sporadic cells in the tissues that demonstrated dye communication.

Adrenal sections were incubated in solutions containing Lucifer Yellow and rhodamine dextran in order to validate gap junction mediated communication in the adrenal zones (Fig. 3-7). Unlike Lucifer Yellow which is readily transferred through connexin 43 gap junctions, the rhodamine dextran molecule (10,000 Da) is gap junction impermeable due to size exclusion (el-Fouly et al., 1989). Only the cells at the cut edge contained rhodamine dextran irrespective of the adrenal zone. In the identitical tissue specimen, extensive Lucifer Yellow dye communication was observed in the inner cortical zonae fasciculata/reticularis. The results from the rhodamine dextran exclusion and the gap junction inhibition assays confirm that the functional intercellular communication observed within the adrenal cortex is mediated by the connexin 43 gap

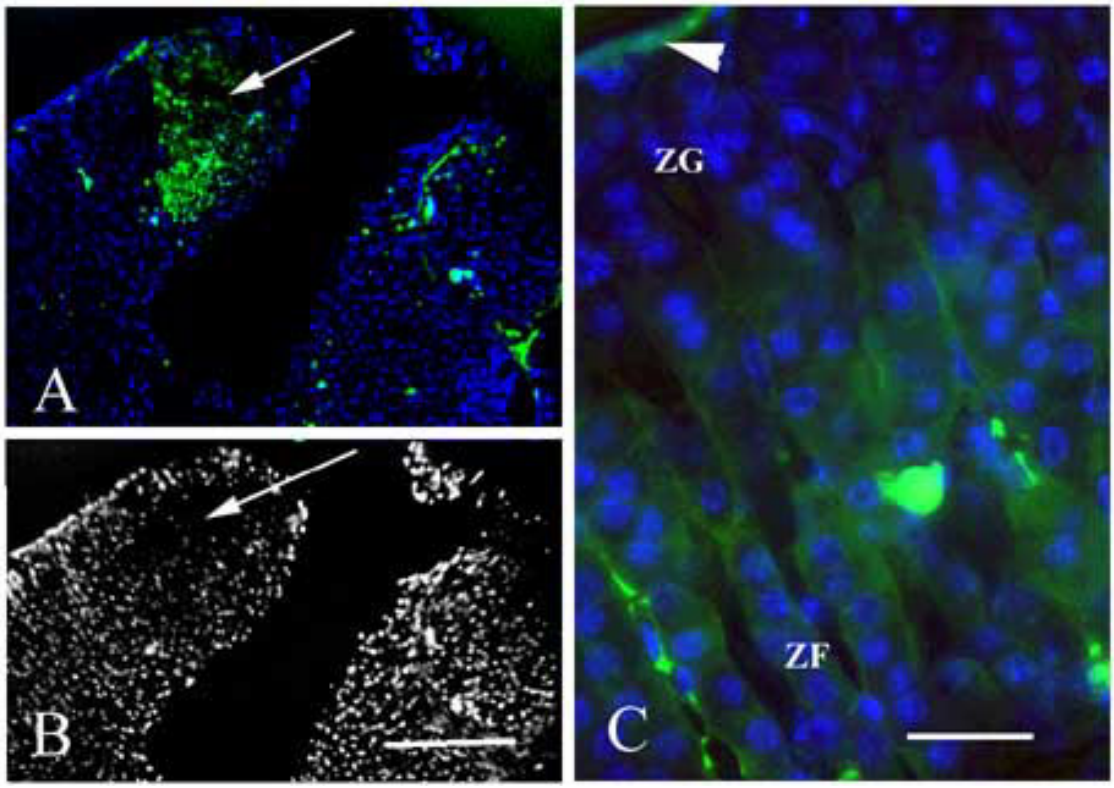

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Figure 3-6 Adrenal cortical Lucifer Yellow dye communication in the presence the gap junction inhibitor oleamide

In the presence of oleamide, Lucifer Yellow dye communication (green) was confined to areas of tissue injury in the adrenal gland, arrow (A). The lack of Hoescht nuclear dye in the tissue specimen demonstrated extensive tissue injury due to the loss of cortical cells, arrow (B). Adrenal specimens exposed to oleamide exhibited an attenuation of intercellular communication in the normally communication competent zona fasciculata (green) (ZF). Arrowhead, adrenal capsule. ZG, zona glomerulosa. Hoescht stained nuclei, blue (A and C). Bar = 80 μm in (A and B). Bar = 30 μm in (C).

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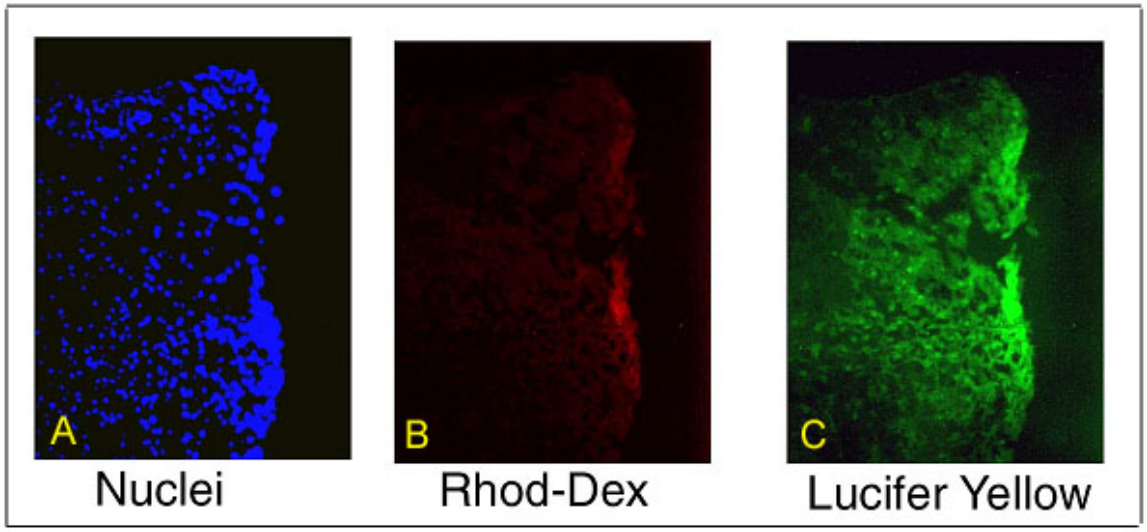

Figure 3-7 Gap junction mediated intercellular communication in the adrenal cortex

Morphology of an adrenal specimen stained with Hoescht nuclear dye (blue, A). Rhodamine dextran (10,000 Da), which is impermeable to gap junctions due to size exclusion, was restricted to the injured cells at the cut edge of the adrenal gland specimen (red, B). Conversely, Lucifer yellow is gap junction permeable (443 Da) and was transferred extensively through the inner cortical cells that are abundant is gap junctions (green, C).

junctions and not as of result of tissue injury.

Next, a cross-species analysis was performed using adrenal glands from the primate, Macaca mulatta, to determine if the relationship between connexin localization and dye communication was species specific. The primate adrenal glands also exhibited a communication profile that is similar to that seen in the murine model. The zona glomerulosa showed few cells capable of communicating the Lucifer Yellow dye to neighboring cells. As seen in the mouse adrenal gland, the ZF/ZR of the primate adrenal gland demonstrates high levels of cell-cell communication as assessed by Lucifer Yellow dye transfer (Fig. 3-8). The dye extends from the cut edge to many cell layers within the inner cortical zones. In accordance with the observations in the mouse adrenal cortex, the cellular coupling in the monkey adrenal gland was confined to the inner cortical zones

3.1. Gap Junction Mediated Communication and Zone Specific Steroid Synthesis

The zonal identity assigned to the different subtypes of steroid producing cells within the different adrenal zones has been traditionally ascribed to zone specific anatomical features. To validate that the observed differences in gap junction expression and cell-cell communication in the adrenal cortex are attributable not only to anatomy but also to zone-specific function, it was necessary to delineate the steroidogenic capacity of communication competent and incompetent cells within the three adrenocorical zones. P_{450} aldosterone synthase is a mitochondrial enzyme, and its expression in the zona glomerulosa definitively identifies the mineralocorticoid synthesizing zona glomerulosa (Denner, et al., 1996; Wotus et al., 1998).

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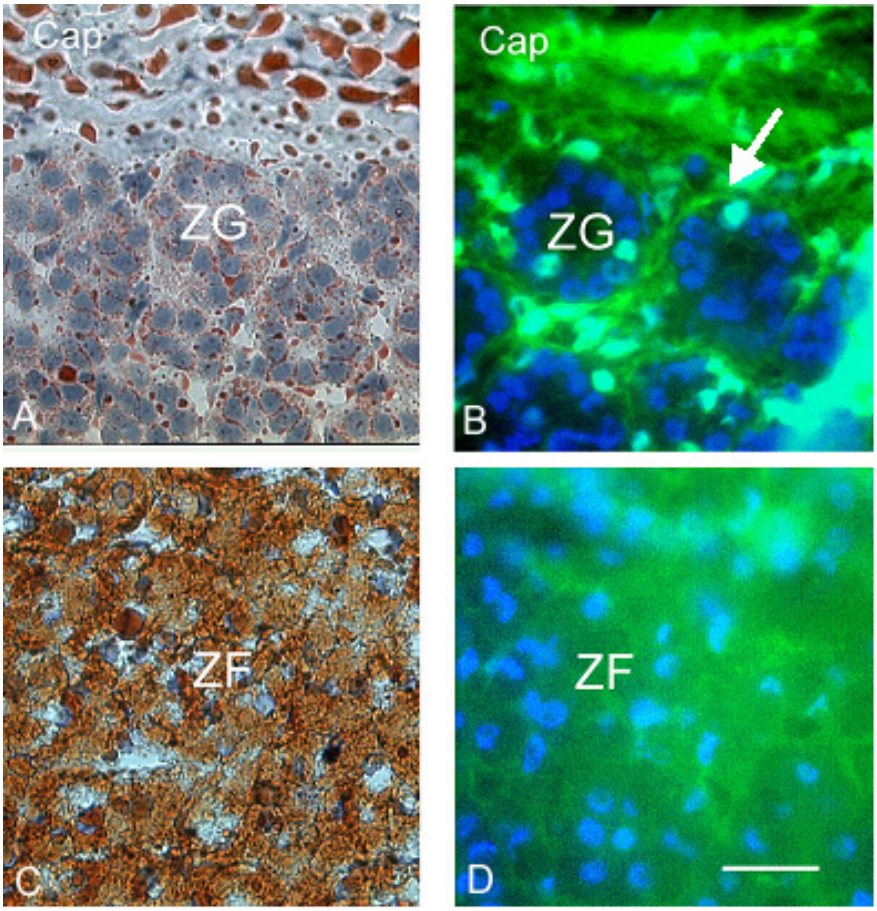

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Figure 3-8 Gap junction mediated communication in the monkey adrenal cortex

Histochemical stain of a monkey adrenal gland with Oil red O (red A, C) and eosin (blue A, C) were used to demonstrate cellular lipid content and adrenal morphology. The pattern of cell-cell communication as demonstrated by Lucifer Yellow staining was identical to that seen in the mouse adrenal gland (B). The parenchymal cells of the zona glomerulosa failed to transfer the Lucifer Yellow dye, which was restricted to the connective tissues in this zone arrow in B. The inner cortical cells of the zona fasciculata transferred the Lucifer Yellow dye indicating abundant intercellular communication in the deeper cortical regions (D). Cap = Adrenal capsule. Bar = 30 μm.

Mitochondrial morphology alone was formally used as one of the primary identifying markers of the three adrenal zones because it is altered under different hormonal and physiological demands (Mitani, et al. 1994). However, the fundamental determinant of adrenal zonation is the synthesis of the steroid enzymes specific to the different zones. The use of immunocytochemical markers directed against specific mitochondrial proteins permits a more concrete determination of the steroidogenic potential of the adrenal zones. This approach validates the zonal identity of cells in the adrenal cortex while eliminating the need to rely solely on morphological features as the determinant for specific cortical identity of cells in the various adrenal zones.

3.2. Zone specific steroid production and communication

The cells of the outer cortical regions beneath the adrenal capsule expressed high levels of the P_{450} aldosterone synthase marker (Fig. 3-9). The P_{450} aldosterone synthase positive cells were typically 4-6 cells layers deep and corresponded histologically to zona glomerulosa cells. P_{450} aldosterone synthase positive cells did not demonstrate the ability to communicate Lucifer Yeloow dye. The connective tissue cells of the adrenal capsule were also absent of the P_{450} aldosterone synthase enzyme according to the immunological staining. The P_{450} aldosterone synthase negative cells were restricted to the deeper cortical regions and exhibited both high levels of connexin 43 and dye communication in contrast to the cells in outer cortex (Fig. 3-10). The inverse relationship between P_{450} aldosterone synthase expression and competency for gap junction mediated communication establishes a strong correlation between communication and adrenal cell function. The whole gland dye communication assay coupled with the staining of a

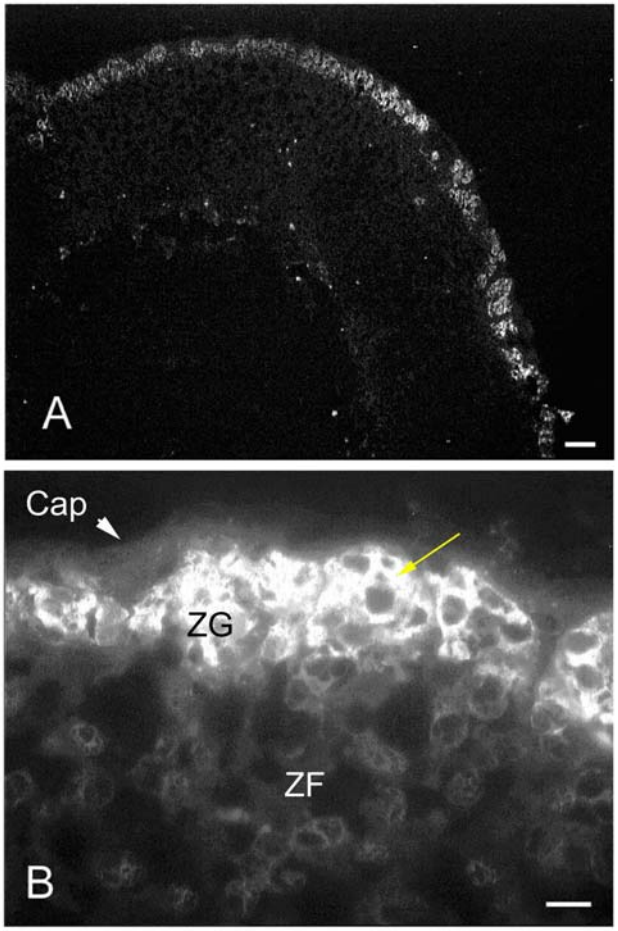

Figure 3-9 Cytochrome P_{450} aldosterone synthase expression in the zona glomerulosa

The cytochrome P_{450} aldosterone synthase (P_{450} aldo) was localized solely in the cells of the zona glomerulosa (ZG). P_{450} aldo is identifiable as a ring of staining in the outermost cells of the adrenal cortex (A). P_{450} aldo is a mitochondrial protein and was expressed intensely in the cytoplasm (yellow arrow) of the zona glomerulosa cells and is absent from the cells in the adrenal capsule (white arrowhead) (B). P_{450} aldo identifies the mineralocorticoids secreting cells of the zona glomerulosa and distinguishes them from the deeper cells of the zonae fasciculata/reticularis (ZF), which express no P_{450} aldo (B). Cap = adrenal capsule. (A) Bar = 50 μm. (B) Bar = 10 μm.

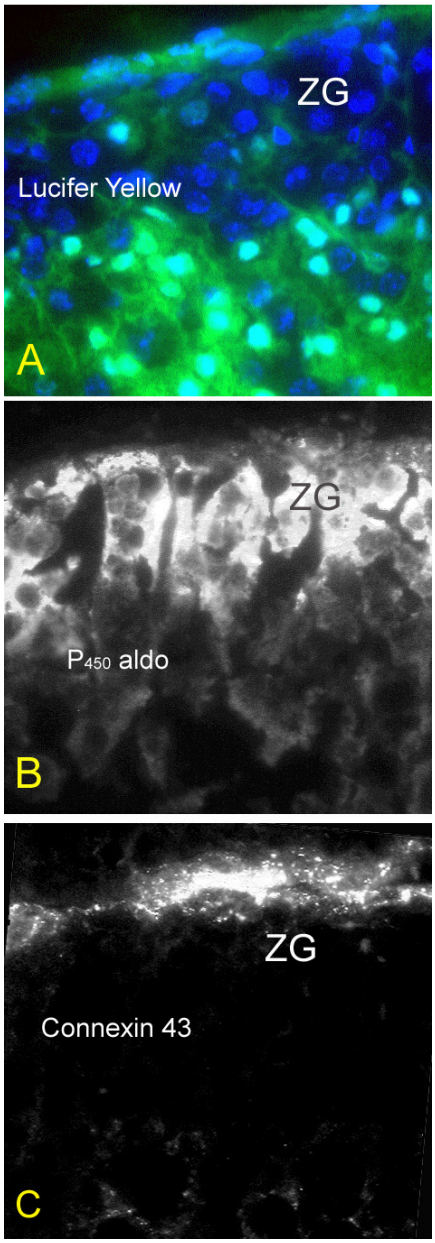

Figure 3-10 Cell-cell communication, gap junction expression and steroid production in the adrenal cortex

A cell-cell communication assay in primary adrenal tissues showed that communication deficient cells of the zona glomerulosa do not transfer the gap junction permeable Lucifer Yellow dye (A), but maintain high levels of the mineralocorticoid synthesizing P_{450} aldosterone synthase (P_{450} aldo) gene (B) and nearly undetectable levels of the connexin 43 protein (C). Note high levels of communication in the sub-glomerulosa cells of the zona fasciculata (A), which produce no P_{450} aldo.

definitive marker for the glomerulosa (P_{450} aldosterone synthase) collectively distinguished the outer cortical zona glomerulosa as distinct in both is steroidogenic and communication potential from that of inner zonae fasciculata/reticularis.

Summary

Connexin 43 gap junction distribution in the adrenal cortex is consistent with the theory that precise regions of intercellular coupling are necessary for basic adrenocortical function. Functional gap junction mediated communication mirrors connexin 43 expression. The zona glomerulosa parenchymal cells express little detectable connexin 43 gap junctions and are incapable of gap junction mediated communication, as demonstrated by their inability to transfer Lucifer Yellow dye. By comparison, the zonae fasciculata/reticularis are highly communication competent and express abundant gap junctions. Furthermore, the absence of dye communication in the zona glomerulosa supports the conclusion that only the connexin 43 isoform is present in the outer cortical cells.

The primary hallmark of the adrenal cortex is its morphological and steroidogenic stratification. The localization of cytochrome P_{450} aldosterone synthase in the communication deficient cells confirmed their zona glomerulosa identity. This zonal variation of dye transfer further suggests an intercellular communication requirement in the glucocorticoid synthesizing zona fasciculata that is absent in the mineralocorticoid producing cells of the zona glomerulosa.

The findings described here also raise questions concerning intercellular communication in normal adrenal homeostasis, such as: 1) Does the alteration of the trophic state of the adrenal gland alter communication patterns facilitated by gap junctions? 2) Are hormonal factors that are responsible for adrenal homeostasis linked to the modulation gap junction mediated

communication? Nevertheless, an intrinsic pattern of connexin 43 distribution in the adrenal cortex further stratifies the adrenal zones along communication boundaries. The demonstration that regional communication differences exist establishes that zonal differences in the adrenal cortex may be related not only to anatomical position but also to the ability to exchange information between the different adrenal zones through gap junctions.

4. CHAPTER 4 CONNEXIN 43 EXPRESSION AND HYPOPHYSECTOMY

The control of tissue homeostasis involves the regulation of intercellular signals through gap junctions (De Feijter et al., 1990; de Feijter-Rupp et al., 1998; Evert et al., 2002; Trosko et al., 1990; Trosko et al., 1998). The modulation of gap junction expression in several endocrine tissues affects both function and hormonal response (Calabrese et al., 2001; Juneja et al., 1999; Meda et al., 1983; Mehta et al., 1986; Stumpel et al., 1998). In the adrenal gland, ACTH is a potent homeostatic regulator and activates second messenger pathways involving Ca^{2+} and cAMP in the zona glomerulosa and zona fasciculata respectively (Gallo-Payet and Payet, 2003; Rainey, 1999). Since intercellular communication is differential in the three cortical zones, it is possible that second messenger signaling plays a critical role in zone specific functions. The zonae fasciculata/reticularis maintain abundant connexin 43 gap junctions and are communication competent in contrast to the relative lack of connexin 43 in the communication deficient zona glomerulosa (Davis, et al, 2002). Therefore, factors which compromise adrenal cortical physiology are hypothesized to alter gap junction mediated cell-cell communication via changes in connexin protein expression and distribution.

The disruption of the hypothalamus-pituitary-adrenal axis (H-P-A) via hypophysectomy (pituitary removal) is known to affect several features of adrenal physiology (Ariyoshi, et al. 1998; Robinson, et al., 1983). After the stimulation by corticotropin releasing hormone (CRH), the pituitary secretes ACTH (Fig. 4-1). ACTH acts on the adrenal gland to regulate a variety of adrenal processes by the activation of several second messenger cascades (Murray and Fletcher, 1984; Vinson, et al., 1989). ACTH is involved in a variety of adrenal cell specific functions including growth control, storage, hormone secretion, cell proliferation and overall glandular

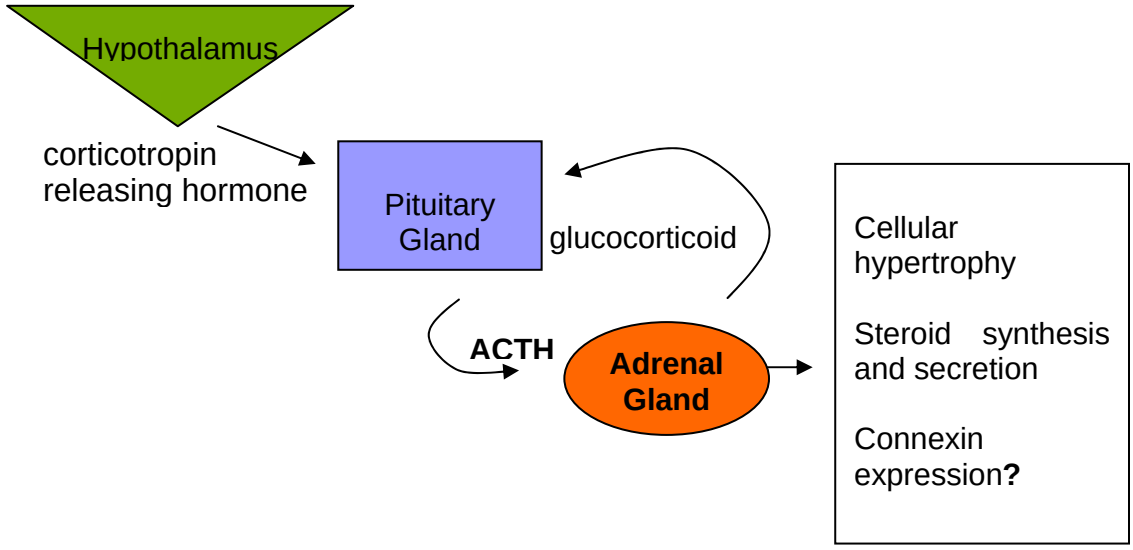

Figure 4-1 ACTH signaling on the adrenal gland via the hypothamus-pituitary-adrenal axis

The hypothalamus releases of the corticotrophin releasing horme (CRH) that stimulates the anterior pituitary to secrete ACTH (adrenocorticotropin hormone). ACTH binds its G-protein coupled receptors on the membrane of the adrenal cortical cells and stimulates a signaling cascade that affects several aspects of adrenal function such as the cortical cell differentiation, cellular hypertrophy and steroid synthesis. The steroid glucocorticoid, whose synthesis is positively affected by ACTH, acts through a negative feedback mechanism to modulate pituitary secretion of ACTH. The effect of ACTH on gap junction expression was postulated as another important end point of ACTH regulation. The relevance of endogenous ACTH on the production and maintenance of adrenal gap junctions is also proposed.

homeostasis (Bornstein, et al., 1990; Ceccatelli, et al., 1995; Migally, 1980; Nickerson and Brownie, 1987). ACTH also stimulates adrenal cell cultures to upregulate gap junction synthesis and increase steroid production while decreasing cell proliferation rates (Murray and Shah, 1998; Shah and Murray, 2001). The specific effects of the loss of endogenous ACTH signaling through hypophysectomy, however, have not been explored in the context of the gap junction mediated communication in the intact adrenal gland. Hypophysectomy allows for the study of adrenal hormonal response *in vivo* by the removing the intrinsic source of ACTH signals.

Although the adrenal cortical zones are exposed to many of the same humoral and neuronal stimuli, they have individual requirements, which necessitates a mechanism for highly zone-specific responses. For example, while ACTH receptors expression within the adrenal cortex is relatively uniform, the action by which ACTH produces different responses in the zona glomerulosa from those in the zona fasciculata is not fully defined (Liaskos et al., 1998; Rainey, 1999; Xia and Wikberg, 1996). With the understanding that ACTH is critical for normal adrenal function and that gap junctions are required for proper maintenance of multiple tissues, an evaluation of the ACTH stimulus in the adrenal cortex is duly warranted. Hypophysectomy then is an efficient means to elucidate the differential hormonal response across the cortical regions while still maintaining the glandular integrity.

Results

The loss of the endogenous ACTH stimulus subsequent to hypophysectomy was typified by changes in gross adrenal morphology and changes in the body mass of hypophysectomized mice when compared to their control littermates. The observed changes and were advanced at 30 days following hypophysectomy and persisted throughout the latest time point measured

following the procedure (110 days) (Fig. 4-2). Hypophysectomized mice exhibited a 20% reduction in body mass at time points greater than 30 days following hypophysectomy. The mouse hypophysectomized adrenal glands exhibited characteristics consistent with previously established features associated with hypophysectomy. Adrenal glands from the hypophysectomized animals were appreciably smaller and the relative cortical thickness was also reduced (Fig. 4-2). Histochemical staining of the adrenal tissue revealed that cells in the adrenal cortex were compacted and atrophic in appearance as demonstrated by the observed increase in the nucleus to cytoplasmic ratio.

Several zone-specific characteristics in the adrenal cortex of hypophysectomized mice were less defined when compared to their control littermates (Fig. 4-2). The cells of the fasciculata lost much of their linear organization and had fewer visible lipid droplets, indicating the loss of steroid synthesis and secretion in adrenal glands from hypophysectomized rodents (Andreis, et al., 1989). Further conformation of a the reduction of zone specific phenotype following hypophysectomy was apparent in the reduction of P_{450} aldosterone synthase expression. The zona glomerulosa specific antigen, P_{450} aldosterone synthase was significantly reduced in the adrenal glands of hypophysectomized mice as as early as 30 days post hypophysectomy (Fig. 4-3). Fluorescent microscopy of Hoescht stained nuclei within the zona fasciculata also showed an appreciable compaction of the nuclei within the inner two-thirds of the adrenal cortex similar to that seen by histochemical staining. A loss of cytoplasmic volume was apparent as the adrenal cells appeared compressed and the internuclear distances were smaller in the hypophysectomized mouse glands compared to control mice.

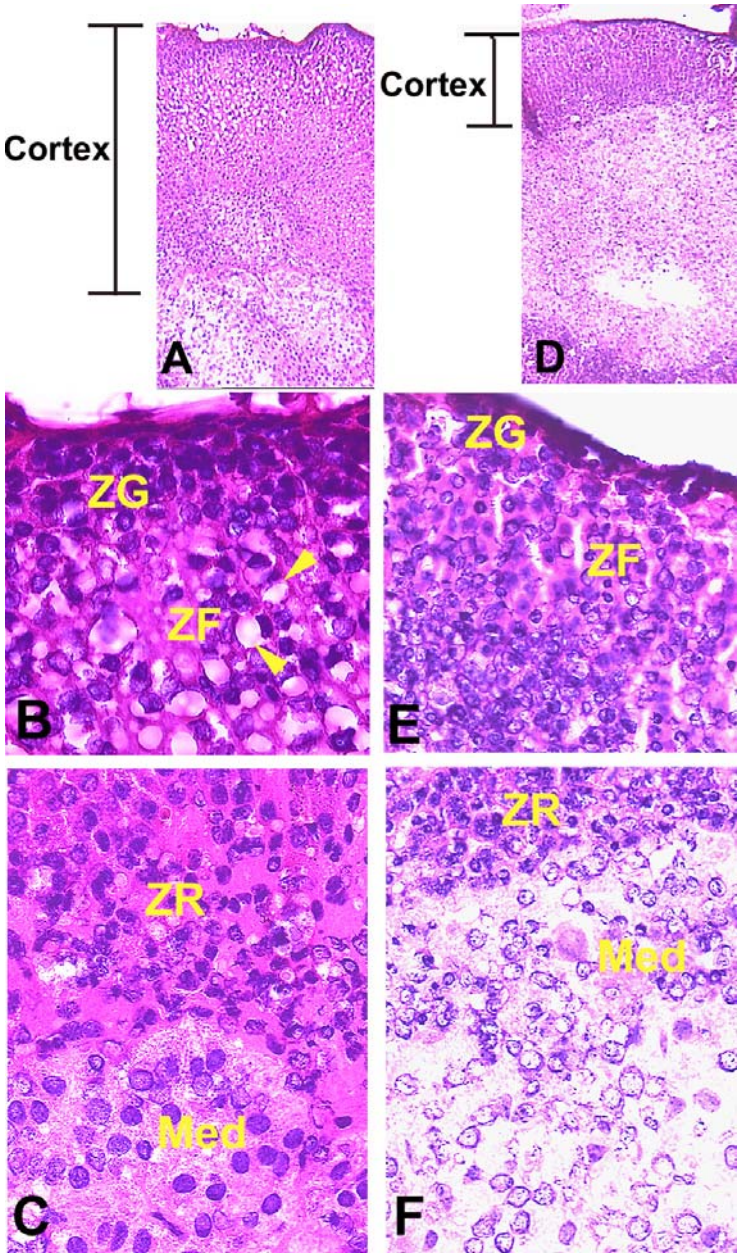

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Figure 4-2 Histochemical staining of the adrenal cortex in normal and hypophysectomized mice with hematoxylin/eosin

The normal adrenal cortex (A- C) is approximately twice the width of that observed in the adrenal glands from hypophysectomized mice 110 days post hypophysectomy (D-F). Normal zonal features such as lipid droplet content (arrowheads, B) in the zona fasciculata and linear morphology (B, C) are diminished in the glands of hypophysectomized mice, which was atrophic in appearance as seen by the lack of cytoplasmic staining and lipid droplets (E, F). ZG = zona glomerulosa, ZF = zona fasciculata, ZR = zona reticularis, Med = Medulla.

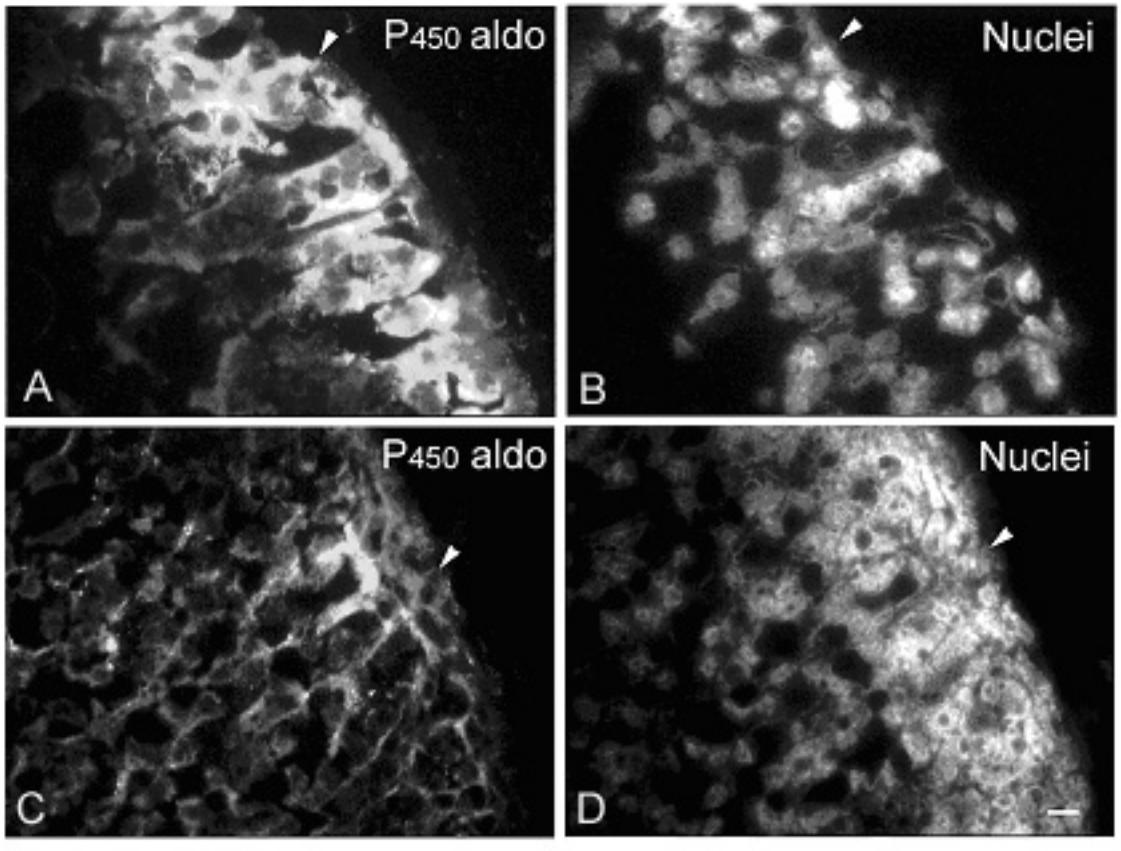

Figure 4-3 Steroidogenic enzyme expression in normal vs. hypophysectomized adrenal glands

Immunocytochemical localization of P_{450} aldosterone synthase (P_{450} aldo) expression in the outermost cells of the adrenal cortex defines the terminal differentiation of the zona glomerulosa (A, C). P_{450} aldo is reduced in the zona glomerulosa adrenal glands from hypophysectomized (post 30 days) mice and in many cases is restricted to small patches of cells in the zona glomerulosa (C). Normal and hypophysectomized adrenal gland were stained with Hoescht nuclear stain (B, D). The decrease in P_{450} aldo positive cells in the adrenal glands of the hypophysectomized is also associated with cortical cell atrophy as demonstrated by the compact nuclear morphology that is typical following the loss of endogenous ACTH production (D). Arrowheads = adrenal capsule. Bar = 10 μm.

To investigate the effect of the ACTH stimulus on the adrenal cortical gap junctions, adrenal glands harvested from hypophysectomized mice were analyzed for immunocytochemical changes in connexin 43 gap junction localization and distribution. The connexin 43 staining pattern was limited to the cortical regions of the zonae fasciculata reticularis (Fig. 4-4). When compared to control specimens, connexin 43 positive cells in ACTH depleted mice were dramatically reduced within the inner two-thirds of the adrenal cortex. Immunolocalized connexin 43 in hypophysectomized mice was restricted to the inner 1/3 of the adrenal cortex in contrast to the abundance of connexin 43 in the outer two-thirds of the cortex in control mice. Gap junction number was measured as a function of connexin 43 particles/per unit area due to difficulty of counting cell nuclei in the compressed cortex of the adrenal glands of hypophysectomized mice (Fig. 4-5). Under these parameters, there was a decrease in gap junctions in the zona fasculata subsequent to hypophysectomy, and an unexpected increase in gap junctions in the zona reticularis at day 33 post hypophysectomy (Fig.4-6). The zona glomerulosa showed no changes in connexin 43 expression following hypophysectomy.

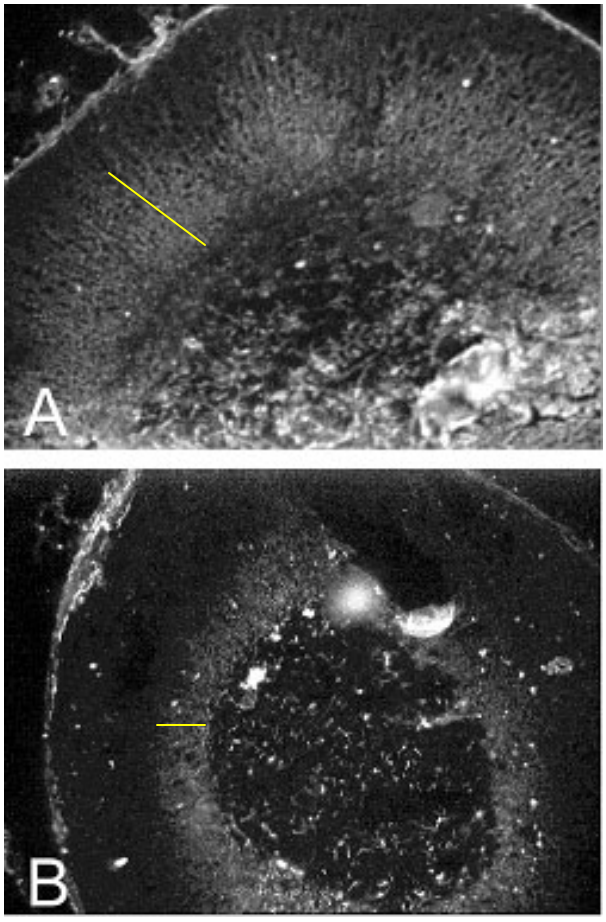

Figure 4-4 Localization of gap junctions in normal and hypopysectomized mouse adrenal gland following hypophysectomy

Immunolocalization of connexin 43 in both normal (A) and hypophysectomized mice (B) 30 days following pituitary removal. The normal adrenal gland maintained connexin 43 over the inner 2/3 of the adrenal cortex (line). In contrast, the distribution of the connexin 43 protein, in glands derived from hypophysectomized mice was diminished as demonstrated by the ring of connexin 43 positive cells in the innermost regions of the adrenal cortex. Yellow lines in A and B indicate the extent of connex 43 positive cells in the cortical regions.

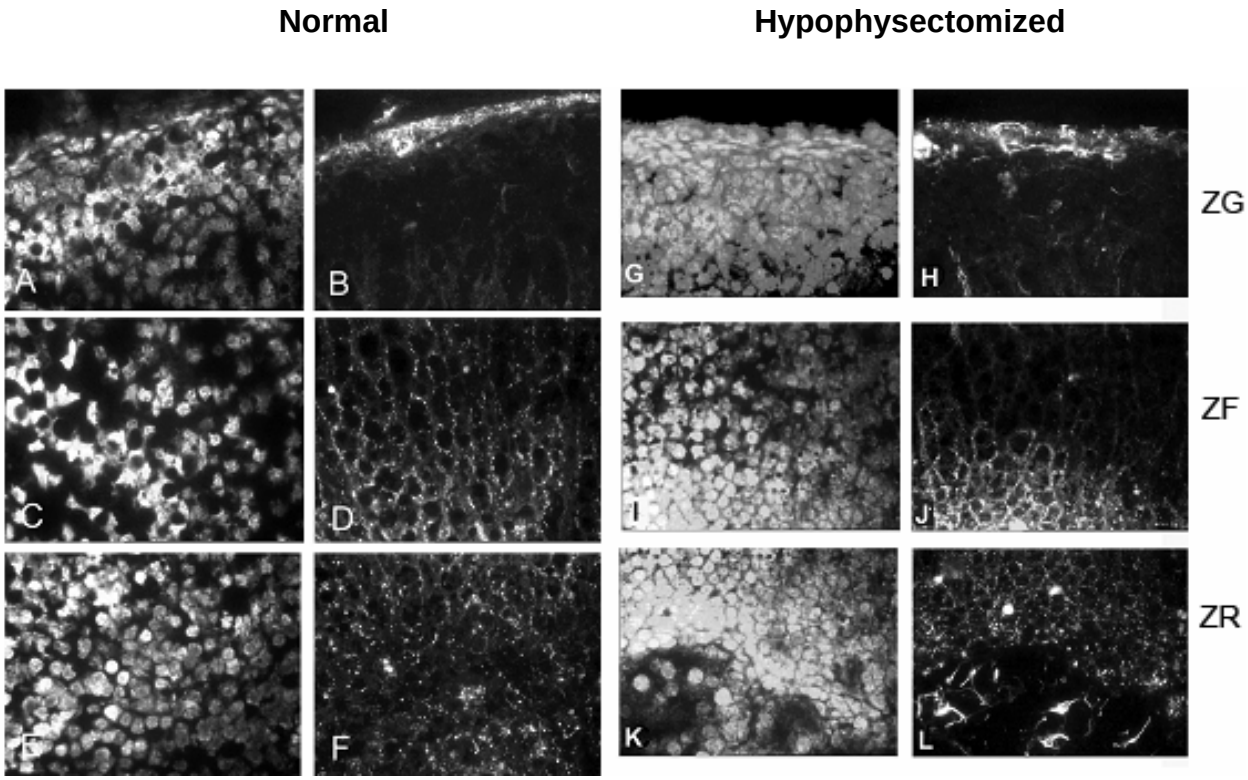

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Figure 4-5 Connexin 43 localization in normal and hypophysectomized mouse adrenal glands

Indirect immunofluorescence of connexin gap junctions in normal (B, D, F) and hypophysectomized (H, J, L) mouse adrenal glands. Normal mouse adrenal glands expressed connexin 43 gap junctions in a distribution that was characteristized by virtually no connexin 43 expression in the outer zona glomerulosa, ZG (B), while connexin 43 expression (visible as punctate fluorescence) is abundant in the inner zonae fasciculata/reticularis, ZF/ZR (D, F). Hypophysectomized mice (33 days post hypophysectomy) had a similar absence of connexin 43 in the ZG that was comparable to the normal mice (H). Hypophysectomized mice experienced a dramatic decrease in connexin 43 in the zona fasciculata that was restricted to the innermost cells of the ZF at the same time point (J). Connexin 43 gap junctions in the zona reticularis appeared to increase slightly (L). Nuclear morphology in normal (A, C, E) and hypophysectomized (G, I, K) adrenal glands was visualized with Hoescht dye. Glandular morphology in the hypophysectomized mice was compressed and atrophic (as assessed by nuclear morphology) in contrast to the normal glands, which had nuclear spacing that allowed identification of individual nuclei in all the cortical zones.

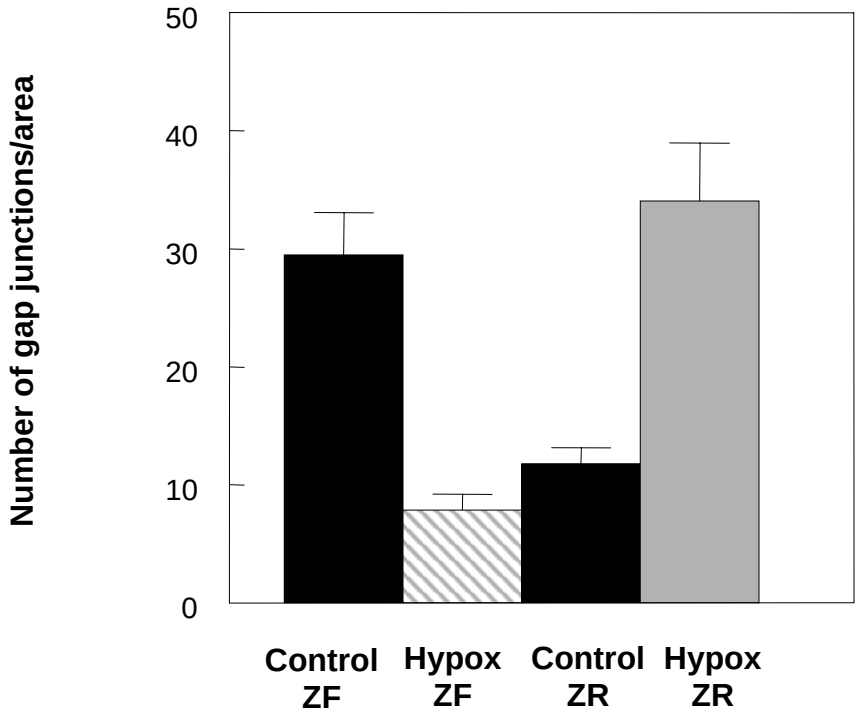

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Figure 4-6 Connexin 43 gap junctions in adrenal cortex post-hypohysectomy

The connexin 43 protein was appreaciably diminished in the zona fasciculata of the adrenal glands examined. In contrast, gap junctions in the zona reticularis were increased 33 days post hypohysectomy. The zona glomerulosa was excluded as virtually no connexin 43 was detectable in this zone. ZF = zona fasciculata. ZR = zona reticularis.

4.1. Effect of exogenous ACTH administration on adrenocortical gap junctions

The loss of the endogenous ACTH by hypophysectomy results in a reduction of connexin 43 from the inner cortical zones of the adrenal gland. The potential stimulatory effect of ACTH on connexin protein levels was investigated by the administration of intraperitoneal injections of ACTH to hypophysectomized mice 64 days following hypophysectomy. Exogenous ACTH treatment of hypophysectomized mice was predicted to increase the expression of connexin 43 gap junctions in the zonae fasciculata/reticularis. ACTH administration was followed by rapid glandular hypertrophy as observed from changes in nuclear morphology 1-3 hrs following the injections (Fig. 4-7). As expected, the hypophysectomized mouse adrenal glands exhibited a marked increase in connexin 43 staining in the zonae fasciculata/reticularis subsequent to the ACTH injections. The increase in connexin 43 gap junctions also occurred 1-3 hrs after ACTH administration and followed the same time course as the morphological changes (Fig. 4-8). Unlike the increase of connexin 43 gap junctions in the zona reticularis at 33 days post hypohysectomy, connexin 43 gap junction number in the zona reticularis of mice 64 days following hypophysectomy exhibited a reduction in connexin 43 expression in both the zonae fasciculata and reticularis. In contrast, hypophysectomized mice that only received saline injections showed no alteration in connexin 43 expression.

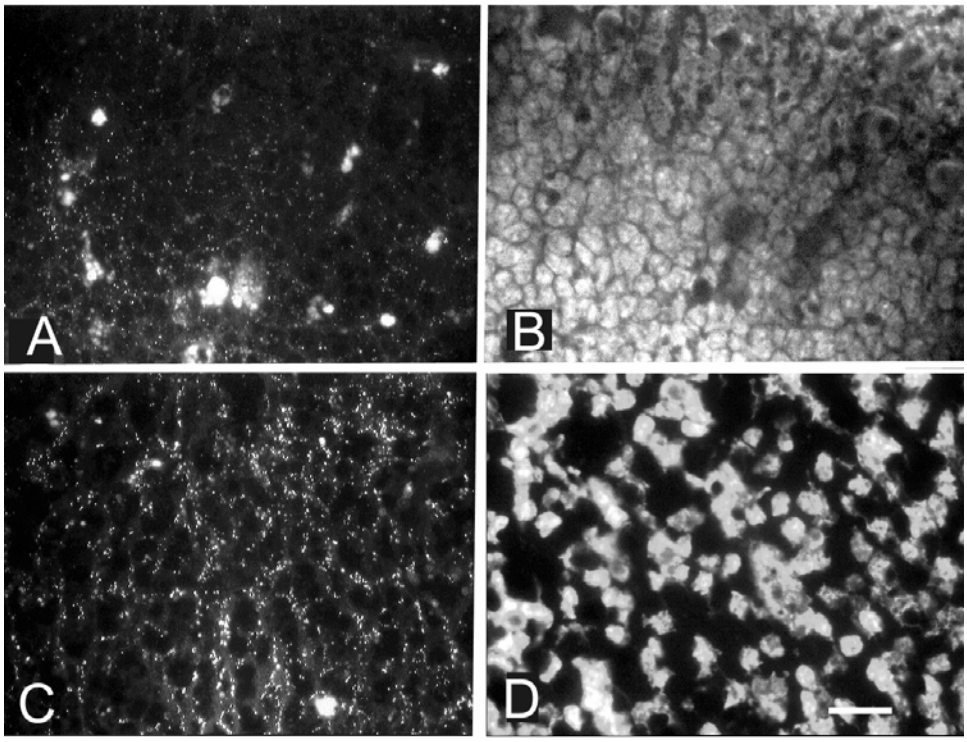

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Figure 4-7Acute effect of ACTH injection in adrenal glands from hypophysectomized mice

Indirect immunofluorescence of connexin 43 expression in hypophysectomized animals (A) is increased 1-3 hrs after receiving intraperitoneal injections of ACTH in the zona fasciculata (C). The hypophysectomized mouse adrenal glands (C) also underwent a change in adrenal morphology that resembled normal zona fasciculata subsequent to ACTH treatment (D). Bar 20 μm.

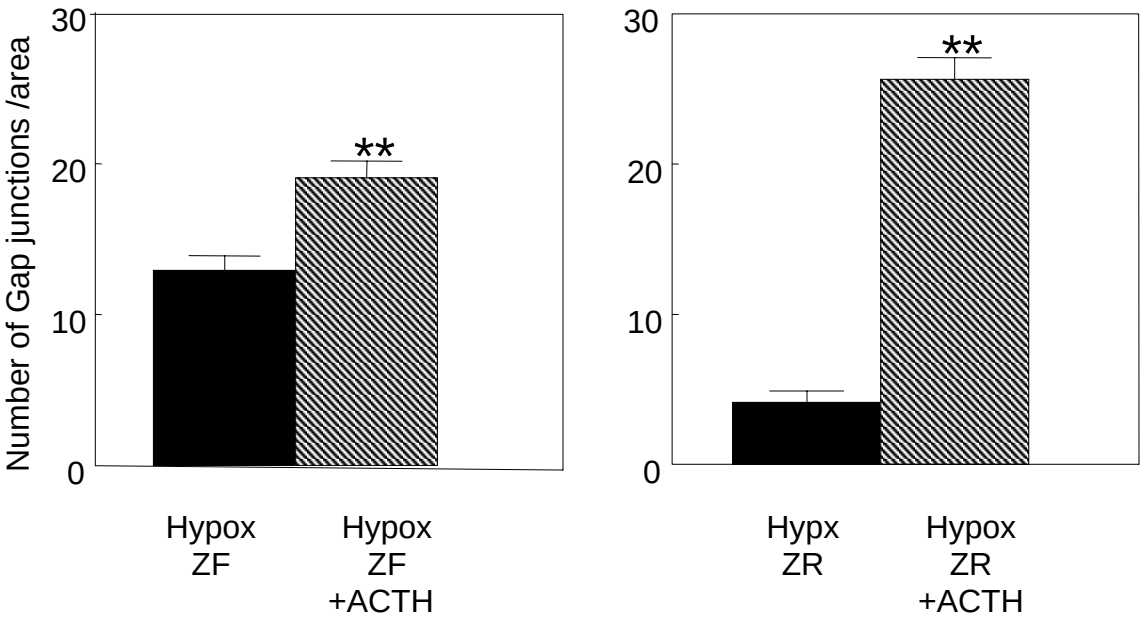

Figure 4-8 Changes in adrenal cortical gap junction number in hypophysectomized mice following ACTH adminstration

The number of connexin 43 gap junctions increased substantially in adrenal glands of hypophysectomized mice (hypox) in the zonae fasciculata and reticularis following intraperitoneal injections of ACTH. The increase in gap junction number in the hypophysectomized mice occurred 1 to 3 hours subsequent to the ACTH treatment**.** Saline treated mice demonstrated no increases in connexin 43 gap junction number following treatment. Asterisks respresent statistically significant difference from saline treatment alone.

Gap junction profile and morphology in normal and connexin 43 deficient mouse

To determine the effect of the absence of gap junctions on adrenal cortical cells, adrenal morphology and zonation were evaluated in connexin 43 deficient mice (Cx 43 -/-) hypophysectomized adrenal glands and suggests a reduced trophic state. In murine models, genetic deletion of connexin 43 results in perinatal lethality several hours following birth (Lo et al., 1997; Reaume et al., 1995). Therefore, adrenal glands were harvested at embryonic day 18. As expected, adrenal glands from Cx 43 -/- mice lacked the punctate connexin 43 staining in the inner cortical spaces that is present in their wildtype littermates (Fig. 4-9). Lipid droplet storage is also diminished in the connexin 43 deficient adrenal glands when compared to the wildtype littermates.

Localization of P_{450} aldosterone synthase was also characterized in Cx 43 -/- adrenal glands to evaluate the integrity of adrenal zonation. While both wildtype (Cx 43 +/+) and Cx 43 -/- adenal glands both expressed the aldosterone synthase enzyme, adrenal glands from connexin 43 deficient mice expressed qualitatively less enzyme and did so in a more stochastic pattern and consisted of reduced expression in small areas and others that were absent of aldosterone synthase (Fig. 4-10). The absence of connexin 43 was also associated with several features that were similar to that seen in the adrenal glands of hypophysectomized mice including glandular atrophy that resulted in a compressed nuclear morphology, decreased lipid storage and a reduction in the expression of the aldosterone synthase enzyme in the outer zona glomerulosa.

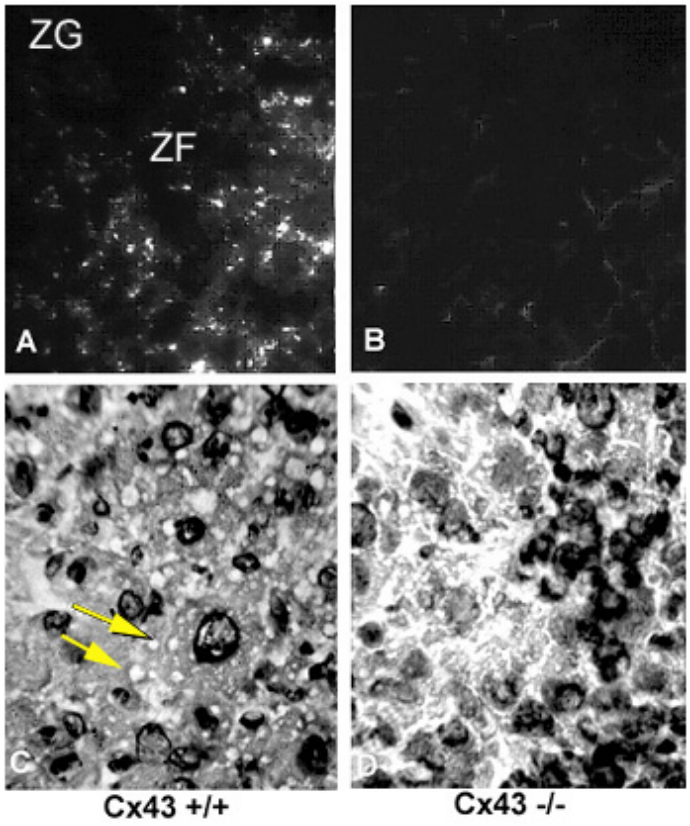

Figure 4-9 Adrenal morphology and connexin 43 expression in wildtype and knockout mouse

Indirect immunofluorescence of connexin 43 expression in wildtype (Cx43 +/+) and connexin 43 deficient mouse (Cx43 -/-). Wildtype neonates demonstrated punctate connexin 43 gap junctions with an adrenocortical distribution typical to that observed in the adult mouse (A). The Cx43 (-/-) mouse expressed no connexin 43 gap junctions (B). Adrenal morphology of the zona fasciculata using hemotoxylin/eosin staining showed abundant lipid droplets (arrows in C) characteristic of the wildtype adrenal glands (C). Adrenal morphology in the Cx43 (-/-) mouse was atrophic in appearance and lipid storage was significantly reduced (D) when compared to the Cx43 +/+ glands. ZG = zona glomerulosa. ZF = zona fasciculata.

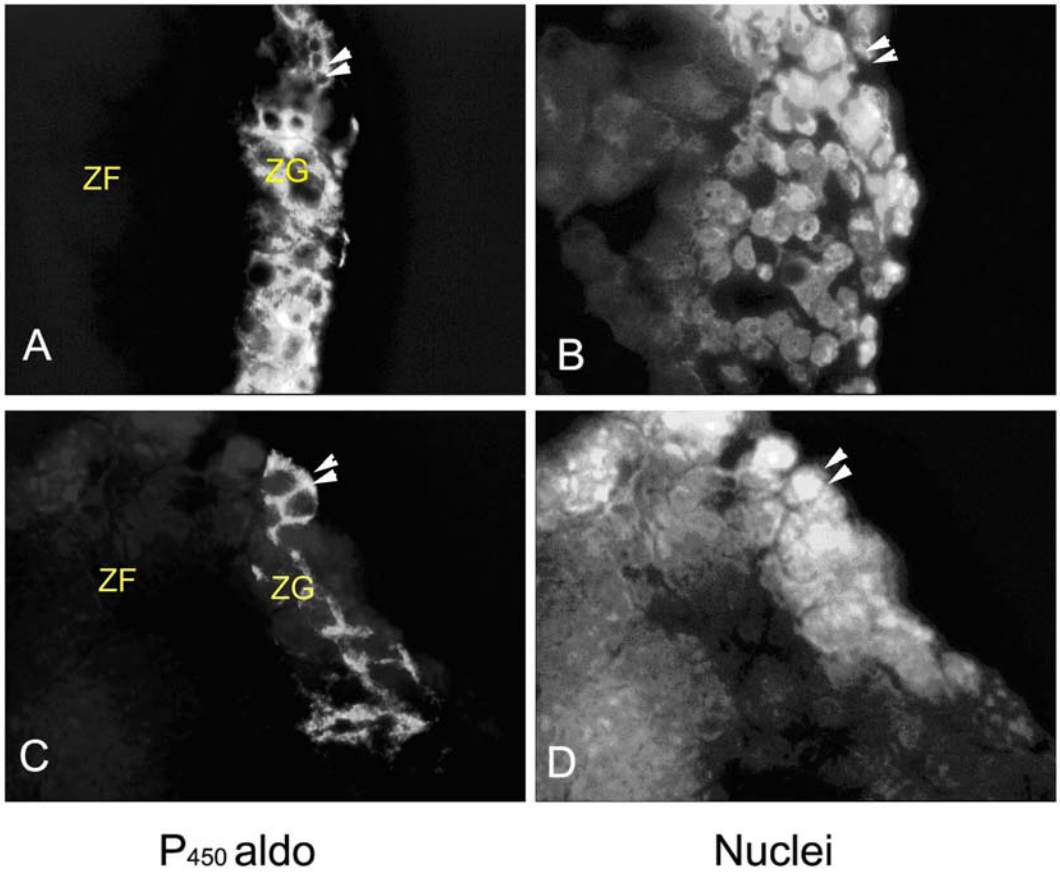

Figure 4-10 Zona glomerulosa specific cytochrome P_{450} aldosterone synthase expression in normal and connexin 43 deficient mouse

Immunocytochemical localization of cytochrome P450 aldosterone synthase (P450 aldo) expression in connexin 43 wildtype mouse (Cx43+/+) is more abundant and occurs with a greater degree of regularity in the zona glomerulosa (A) than that exhibited in the connexin 43 deficient mouse (Cx43-/-) (C). A comparison of nuclear morphology demonstrated that internuclear spaces in the Cx43-/- mouse are reduced (D), indicating a loss of cytoplasmic volume and glandular atrophy when compared to the Cx 43 (+/+) adrenal gland (B). Double arrow = adrenal capsule. B and D = Hoescht nuclear stain.

Summary

The dependence of the hypothalamus-pituitary-adrenal axis on adrenal physiology was examined by hypophysectomy to test the hypothesis that adrenal gap junctions are hormonally responsive and dependent on adrenal trophic state. The loss of endogenous ACTH signaling as a result of hypophysectomy negatively affected adrenal gland gap junction abundance with a concomitant change in adrenal morphology and an apparent reduction in adrenal trophic state. The overall cortical area was reduced in adrenal glands from hypophysectomized mice and the zonation of the cortex were less defined as evidenced by the loss of typical lipid storage droplets in the zona fasciculata, cortical atrophy, and a reduced expression of P_{450} aldosterone synthase in the zona glomerulosa.

In the adrenal glands from intact animals, connexin 43 gap junction plaques were detected in the cells of the zonae fasciculata reticularis, while few gap junctions were identified in the zona glomerulosa. The atrophy associated with hypophysectomy was detected concomitantly with a loss of connexin 43 plaques from zonae fasciculata and reticularis. There was a time dependent loss of the connexin 43 gap junctions in the adrenal cortex that was pronounced at 30 days following hypophysectomy and that persisted through subsequent time points. The administration of exogenous ACTH to the hypophysectomized mice could increase the connexin 43 gap junctions levels in hypophysectomized mice. The restoration of the connexin 43 plaques was also correlated with glandular hypertrophy indicative of increased glandular function.

Adrenal glands from connexin 43 deficient mice were also found to possess several features present in the hypophysectomized mice. The adrenal cortex from connexin 43 deficient mice was atrophic in appearance and had reduced lipid droplet storage in which P_{450} aldosterone

synthase in the glomerulosa cells was reduced. The two experimental conditions, namely hypophysectomy and genetic deletion of connexin 43, provide empirical evidence for the necessity of gap junction mediated communication in adrenal gland function. The loss of native adrenal gap junction expression in connexin 43 deficient mice is associated with diminished expression of aldosterone synthase, the loss of lipid storage droplets and glandular atrophy similar to that observed in hypophysectomized mice. A reduction of glandular features associated with active adrenal function such as lipid droplet storage and steroid enzyme expression (P_{450} aldosterone synthase) in both the connexin 43 deficient and hypophysectomized mice suggests a physiological link that is hormonally dependent between gap junction distribution and expression and adrenal cortical function.

5. CHAPTER 5 GAP JUNCTION PHENOTYPE IN ADRENAL NEOPLASMS

In adrenal tumors, connexin expression was evaluated to determine whether or not gap junction expression was compromised in human adrenal neoplasms. The rationale is that the abrogation of adrenal homeostatic regulation would also result in the disruption of gap junction protein expression and in turn cell-cell communication involved in adrenal integrity. The disruption of connexin proteins has been investigated in several tumor types and is associated with aberrant proliferation accompanied by a loss of a differentiated phenotype in neoplastic tissues (Chang et al., 2000; Holder et al., 1993; Mehta et al., 1986; Vozzi et al., 1995; Vultur et al., 2003; Yamasaki, 1995). In contrast, gap junctions introduced into neoplastic cells have proven to be tumor suppressive and able to revert some features of neoplastic phenotypes such as aberant proliferation and expression of tissue specific genes (King et al., 2002; Mesnil et al., 1994; Naus et al., 2000).

The malignancy of human adrenal masses is often a difficult but necessary clinical determination as treatment for adrenal tumors varies with malignant potential. There is currently no definitive marker that differentiates benign from metastatic adrenal masses (Cook and Loriaux, 1997). Therefore, the availability of a marker that determines the neoplastic state of adrenal tumors would prove to be a useful tool aiding in both diagnosis and treatment. We hypothesize that connexin 43 expression might serve as a viable marker of tumor grade. This study is predicated on studies of the mitotic indices of cells *in vitro* and *in vivo,* which have shown that the proliferation rates of lung and adrenal derived cells cultures is inversely proportional to connexin abundance (Abraham et al., 2001; Oyoyo et al., 1997; Shah and Murray, 2001). Thus, regulatory signals that are normally transmitted via gap junctions may be

lost or altered during neoplastic growth. It is hypothesized here that aggressively dividing cells of human adrenal tumors would experience a perturbation of connexin expression.

Results

Adrenal glands were donated by patients undergoing nephroctomy or patients who presented signs of adrenal dysfunction and required adrenalectomy. Of the isolated adrenal glands, 3 were normal, 3 were adenomatous and 6 were metastatic carcinomas of indefinite hormonal output. The presence or absence of malignancy was assessed by several established pathological criteria by Dr. Stephan Bornstein (Burghardt and Anderson, 1979; van Slooten, et al., 1985; Weiss, et al., 1984) (see Chapter 2). The adrenal tissues were characterized as: normal, adenoma (benign) or carcinoma (metastatic).

The immunocytochemical profile of connexin 43 distribution in the gland was distinctive in each group of adrenal tissues evaluated. The adrenal tissues were tested for the expression of connexins 26, 32 and 43 as these are the predominant connexin isoforms expressed in several endocrine organs such as the pancreas, liver and thyroid gland (Meda et al., 1993). None of the tumor specimens examined expressed either connexin 26 or 32 in any part of the adrenal cortex. In the normal human adrenal tissue examined, only the connexin 43 isoform was expressed in adrenocortical cells. Gap junction staining was visible as puncta, associated with the cellular borders of the adrenal cortical cells (Fig. 5-1). The expression pattern, however, was variable within the cortical regions consitent with the distribution of connexin 43 that has been previously documented in rodents and bovine adrenal tissues (Murray and Pharrams, 1997). Abundant gap junctions were observed in the connective tissues of the adrenal capsule and to a far smaller degree in the connective tissue trabeculae, which project into the zona glomerulosa.

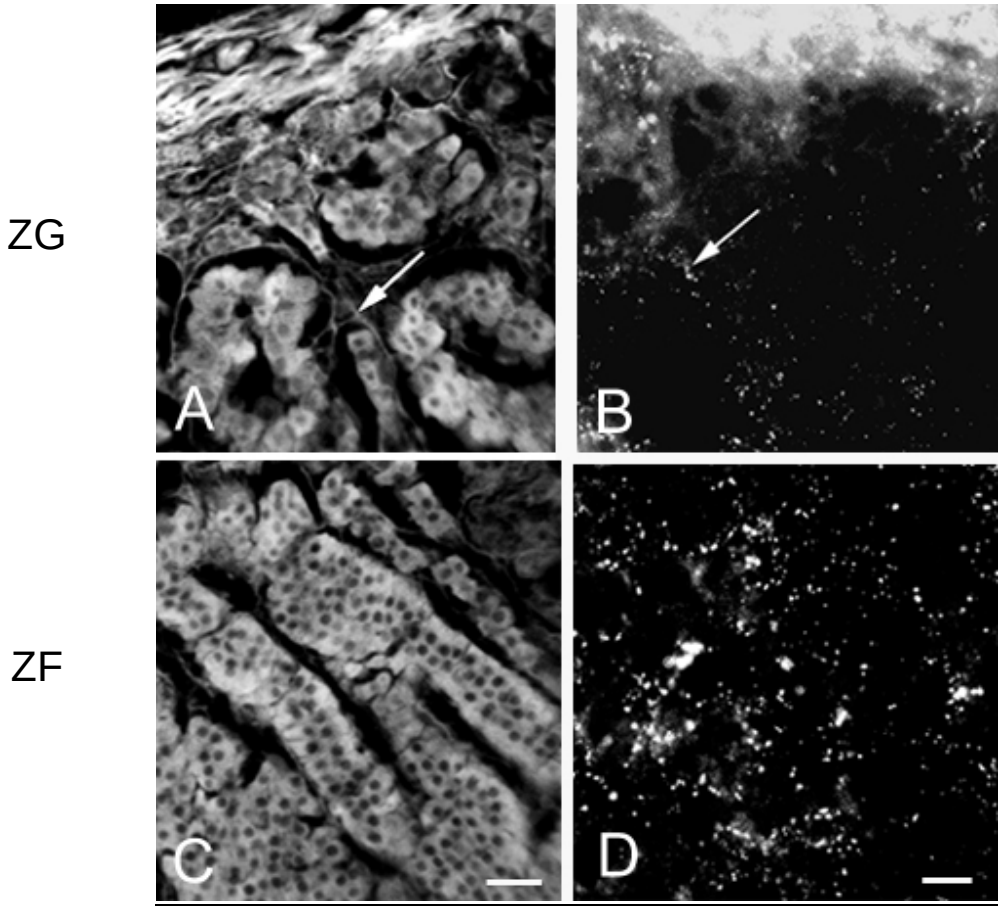

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Figure 5-1 Gap Junction phenotype in the human adrenal gland

Hematoxylin/eosin stain of the human adrenal gland viewed with fluorescent optics (A, C) displaying the morphology of the adrenal capsule, zona glomerulosa, ZG (A) and the zona fasciculata, ZF (C). Indirect immunofluorescence using a connexin 43 specific antibody demonstrates the punctate staining typical of gap junctions (B, D). In the zona glomerulosa, connexin 43 is sparse and restricted to the connective tissue and in the adrenal capsule and the connective tissue extension into the ZG (arrows in A and B). The zona fasciculata exhibited abundant connexin 43 gap junctions in the parenchymal cells. Bar = 25 μm.

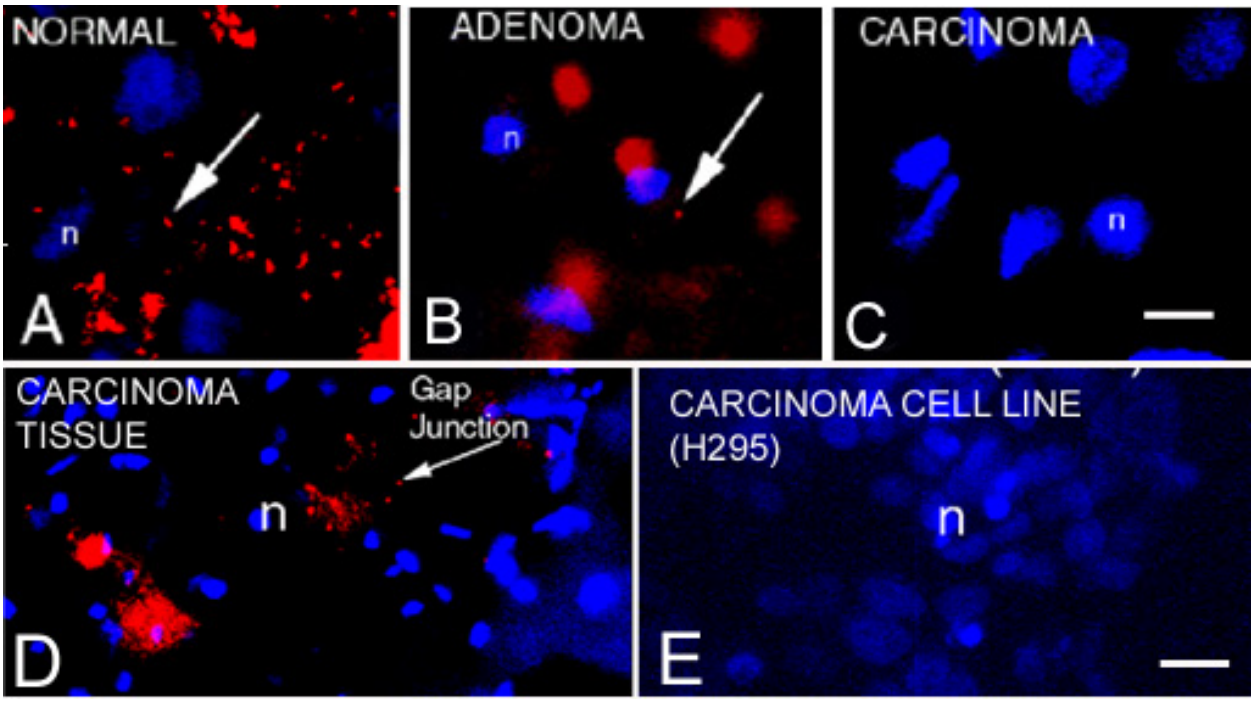

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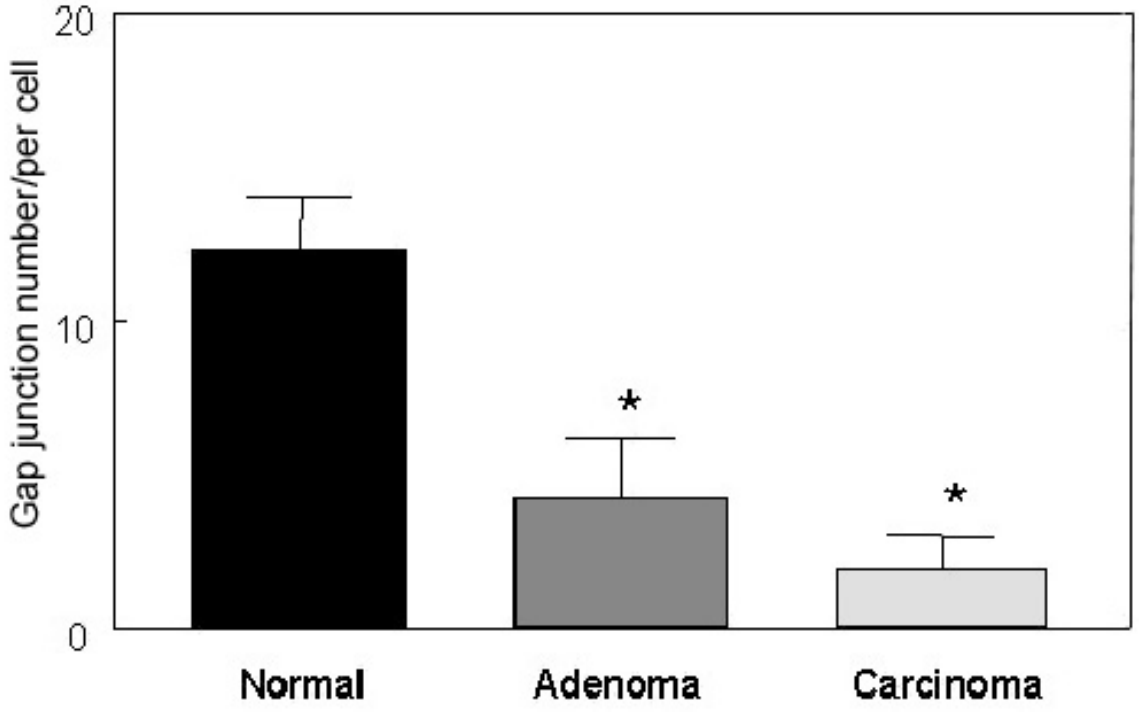
Figure 5-2 Gap junction distrbution in normal and neoplastic adrenal tissues and cells

Immunocytochemical localization of adrenal gap junctions using a connexin 43 specific antibody demonstrated the variable gap junction expression between normal and neoplastic tissues in the zona fasciculata. Normal tissues were abundant in connexin 43 gap junctions (red puncta, arrow A). Benign adenomas expressed less punctate connexin 43 staining that was typical in normal tissue as well as an increased incidence of large, diffuse connexin 43 positive packets. (B). Malignant carcinomas expressed very few identifiable connexin 43 gap junctions (C). When viewed at a lower magnification, a sporadic expression pattern of connexin 43 gap junctions was observed in carcinoma tissue (red puncta, arrow D). The human adrenal carcinoma cell line (H295) is devoid of any connexin 43 gap junctions (E). N= blue Hoescht stained nuclei (blue). Bar = 25 μm. (E) Bar = 30 μm.

A comparison of gap junction expression in normal and neoplastic glands revealed that connexin 43 expression was most abundant in the inner cortex, restricting most of the connexin 43 gap junctions to areas which have been extensively documented, namely the zonae fasciculata and reticularis (Murray et al., 1995). Adenomatous adrenal lesions exhibited a diminished connexin 43 profile in both the zonae fasciculata and reticularis when compared to controls glands. There was also an increased incidence of aberant connexin 43 staining in the adenomatous tissues that is inconsistent with typical punctate gap junctions profile in normal tissue (Fig. 5-2).

The most severe reduction of connexin 43 protein gap junctions was observed in the metastatic tissues of the adrenal carcinomas. Adrenal carcinoma specimens were devoid of over ~90% of the connexin 43 typical in control glands (Fig. 5-3). The staining pattern of connexin 43 was uneven and clustered unlike the even distribution seen in the normal glands or less compromised neoplastic tissues. Using nuclear morphology as a determinant of adrenal integrity, severe disorganization of the adrenal gland was observed in the carcinomas. Many cells were multinucleated and exhibited gross morphological irregularity unlike the defined zonation seen in normal adrenal tissue. The number of connexin 43 gap junctions in the normal, adenomatous and carcinoma tissues was expressed over defined ranges in each of the tissue types evaluated (Fig. 5-3). Normal tissue expressed nearly three times the amount of connexin 43 gap junctions (gj) per cell (13.8 gj/cell $\pm$ 1.9 $_{SEM}$) while the adenoma expressed 4.16 gj/cell $\pm$ 1.17 $_{SEM}$. The average number of gap junctions in the adrenal carcinoma was 1.42 gj/cell $\pm$ 0.58 $_{SEM}$.



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Reproduced with the permission of the Endocrine Society Murray, S. A., Davis, K. T., Fishman, L. M. and Bornstein, S. R. (2000) Journal of Clinical Endocrionology & Metabolism. *Alpha1 connexin 43 gap junctions are decreased in human adrenocortical tumors.* 85 (2)- 890-5.

Figure 5-3 Gap junction number in normal adrenal tissues and adrenal tumors

Each of the three adrenal tissue types examined expressed a distinct range of connexin 43 gap junctions per cell. Normal adrenal tissue is abundant in connexin 43 gap junctions, while benign (adenomas) and metastatic (carcinomas) lesions express appreciably fewer gap junctions. Asterisk denotes statistically significant difference from normal.

Summary

Normal adrenal glands expressed connexin 43 gap junctions with a distribution similar to that found in other mammals. Typically, connexin 43 gap junctions were abundant in the zonae fasiciculata/reticularis, while very few were present in the zona glomerulosa. Neoplastic tissues were reduced in connexin 43 expression in the inner cortical zonae fasciculata/reticularis when compared to normal adrenal glands. Connexin 43 gap junctions were decreased by 30% in benign adenamotous tissue. The metastatic carcinomas had approximately one tenth the amount of connexin 43 gap junctions present in normal tissues. The distinct ranges of gap junctions expression in normal adrenal glands compared to either benign or malignant adrenal specimens, would suggest that connexin 43 expression alone may be used as an index to delineate between normal and the different subtypes of neoplastic adrenal tissues. The identification of gap junctions in adrenal specimens may prove to be a more definitive marker of tumor grade than those currently available.

6. CHAPTER 6 DISCUSSION

The fundamental hypothesis presented throughout these studies is that basic adrenal function involves gap junction mediated communication in the intact adrenal gland. The highly stratified tissue of the adrenal cortex, in which morphological, functional and proliferative differences are restricted to the various sub-populations of the three cortical zones, is an informative system in which to study gap junction distribution and cell-cell communication. A long standing contention is that the differential regulation of the adrenal cortical zones is dictated by physiological demands necessary for homeostasis (Engeland, 1999; Lehoux, et al., 1997; Tremblay, et al., 1992). Few studies have addressed the physiological importance for gap junctions in the adrenal cortex. However, we find that alterations in normal connexin distribution and abundance are coincident with changes in adrenal trophic conditions (Davis et al., 2000). Moreover, connexin 43 protein expression and functional gap junction mediated communication in the adrenal cortex parallel functional differences of the adrenal zones (Davis, et al., 2002). Therefore, the precise expression of connexin 43 in normal adrenal cortical function is potentially required for the maintenance of adrenal homeostasis.

6.1. Dye communication (communication landscape in the adrenal gland)

The dye communication experiments demonstrated for the first time that adrenal cortical gap junctions are in fact capable of functional gap junction mediated intercellular communication in the intact gland (Davis, et al., 2002). The literature illustrating the relationship between adrenal gap junctions and cell-cell communication is predominantly based on dispersed cells in

culture. The comparison between intercellular communication *in vivo* and *in vitro* is an important consideration in terms of the heterogeneous cells of the adrenal cortex, in which zonal differentiation is strongly influenced by changes in hormonal and physiological conditions (Andreis, et al., 1989; Rebuffat, et al., 1991). The abundance of connexin 43 gap junctions and intercellular coupling in the zonae fasciculata/reticularis compared to the paucity of gap junction mediated communication in the zona glomerulosa effectively establishes distinct regions where cell-cell communication is more prevalent. While the precise identity of the endogenous signals transmitted through the gap junction channels cannot be discerned from Lucifer Yellow dye transfer, the abundance of functional cell-cell communication within the inner cortex suggests a critical role for adrenal gap junctions in the regions where it is pronounced. Additionally, the demonstration that the zona glomerulosa specific enzyme, cytochrome P_{450} aldosterone synthase, is present in communication deficient cells of the zona glomerulosa further supports the theory that functional differences across the adrenal cortex are reflected in differences in intercellular communication.

In this context, the rigid maintenance of distinct permeabilities of intercellular signals within the adrenal zones could impact mineralocorticoid and glucocorticoid production in the zona glomerulosa and zona fasciculata respectively. ACTH binding to its receptors in the adrenal cortex does not account fully for the differential action of ACTH in the cortex. ACTH receptors have been identified within each of the three cortical zones, yet a robust glucocorticoid secretion is characteristic of only the zona fasculata (James, 1992; Vinson, et al., 1989). An emergent property of adrenal function can be proposed in which circulating adrenocorticotropin hormone (ACTH) acts to increase the number and distribution of connexin 43 and thereby facilitate cell-cell communication. Several investigators have established that there are divergent

signaling mechanisms involved in steroidogenesis in cells resident in the different adrenal zones (Bird, et al., 1998b; Rainey, et al., 2002). For example, stimulation of ACTH responsive adrenal cortical cells *in vitro* promotes a concomitant increase in connexin 43 expression and steroid production (Murray and Shah, 1998; Shah and Murray, 2001). It has also been demonstrated that adrenal cells increase their synthesis and intercellular transfer of cAMP subsequent to exogenous ACTH stimulus (Hyatt, et al., 1980; Murray and Fletcher, 1984). Moreover, the observation that the adrenal cells utilize an array of gap junction permeable second messengers such as calcium transients, inositol triphosphate and cAMP (Bird, et al., 1998b) would suggests a need to transfer and coordinate these signals differentially in the inner adrenal cortex. Another potential outcome of the zone specific connexin 43 expression may be related to the metabolic requirements of the cortical tissues. *In vitro* studies evaluating the transfer of endogenous metabolites between gap junction coupled tumor cells showed that connexin 43 channels preferentially transmit ATP and glucose than did connexin 32 (Goldberg et al., 1998; Goldberg et al., 2002). The distinct permeability of connexin 43 to metabolites and second messengers may be involved in processes unique to the zona fasciculata/reticularis.

The cells between the zona glomerulosa and zona fasciculata exhibit an intermidiate level of communication when compared to those deeper in the cortex. It is likely that this region represents an intermediate zone, often reported as a zona intermedia (Mitani, et al., 1994). A cell line consistent with the zona intermedia phenotype has been isolated from the adrenal cortex of rats expressing a conditional SV 40 T antigen mutant. Under permissive conditions the cells do not express any of the terminal enzymes critical for the production of either mineralocorticoids or glucocorticoids, which would confer either a zona glomerulosa or fasciculata identity, respectively. However, they can be stimulated to differentiate into cells expressing a zona

fasciculata like phenotype (Mitani, et al., 2003). It would be extremely informative to determine the gap junction phenotype of the zona intermedia cells and if varying gap junction expression in these cells would affect the intercellular communication and differentiation potential.

Several reports in the literature demonstrate that hormonal stimulation of cortical cells promotes their zone specific phenotypes (Mazzocchi, et al., 1986; Ogishima et al., 1992). Vinson *et al* have proposed that hormonal signals result in morphogenic and electrochemical gradients in the adrenal cortex. A morphogenic gradient would provide adrenal cortical cells with positional information that would promote zone specific steroidogenesis (Raza, et al., 1998; Vinson, et al., 1998). The differential communication and distribution of connexin 43 in the adrenal gland may well be involved in establishing its zonal architecture. Coupled with the observation that proper connexin expression is critical for certain cellular and developmental processes (Bauer et al., 2002; Liao et al., 2001), it is reasonable to assume that a similar mechanism is at work in the adrenal gland, which maintains both regional expression of connexin 43, functional intercellular communication and zone specific cellular functions.

6.2. Hypophysectomy

The loss of connexin 43 gap junctions in the adrenal cortex subsequent to hypophysectomy indicates a role ACTH driven hormonal signaling in a normal adrenal phenotype and gap junction expression *in vivo*. Physiologically, such an organization may serve to maintain the specific steroidogenic functions of the adrenal cells by the transmittal of signals through gap junctions. These results are in direct support of earlier electron microscopic studies describing an increase of cortical gap junctions that preceded glucocorticoid surges in fetal rodent and rabbit adrenal cortices. Using freeze fracture and thin layer electron microscopy, fetal

mouse, rat and rabbit adrenal cortices at day 16, 17 and 20, respectively were shown to form gap junction plaques in steroidogenically inactive cells of the zona fasciculata. Following their initial appearance, the number of gap junctions increased achieving maximal levels simultaneously with increased glucocorticoid production (Decker, 1981). In this thesis, connexin 43 was identified as a hormonally responsive gap junction species native to adrenal cortical tissues.

The relationship between hormonal responsiveness and gap junction expression is also supported by *in vitro* studies. ACTH treated adrenal cell cultures expressing connexin 43 synthesize higher levels of connexin 43 gap junctions and increase steroid output when compared to unstimulated populations (Decker et al., 1978; Munari-Silem et al., 1995; Shah and Murray, 2001). In tissues such as bone, connexin 43 expression in osteoblasts is regulated by parathyroid hormone and prostaglandin E2 stimuli by transcriptional upregulation and mobilization of pre-existing connexin 43 pools to the plasma membrane (Civitelli et al., 1998). The respective endocrine and exocrine function of pancreatic islet and acinar cells are regulated by the proper expression of connexin 43 in islet cells and connexins 32 and 26 in pancreatic acinar cells in response to several secretagogues (Bruzzone et al., 1987; Meda, 1996a; Meda, 1996b; Meda et al., 1987). The studies of hypophysectomized mice presented here further show that a relationship between hormonal regulation of gap junctions and organ function exists.

As hypothesized, ACTH preferentially alters connexin expression in the glucocorticoid producing cells of the zona fasciculata/reticularis. This finding suggests a requirement for gap junction mediated communication in the inner cortex that is not present in the zona glomerulosa as gap junctions were not observed in these cells under any conditions assayed. Yet, the cytochrome P_{450} aldosterone synthase expression characteristic of the zona glomerulosa is also

compromised in hypophysectomized glands. The parenchymal cells of the zona glomerulosa, which are responsible for mineralocorticoid synthesis, are responsive to ACTH as well. However, the second messenger pathway involved in steroid synthesis in the zona glomerulosa differs from that proposed for glucocorticoid production in the zona fasciculata/reticularis. Several investigators have described a calcium dependent mechanism that is necessary for angiotensin II stimulated aldosterone production. The zona glomerulosa sigaling cascade differs from the cAMP mediated mechanism of steroid production described in the zonae fasciculata/reticularis (Bird, et al., 1998a; Clark, et al., 1992; Vinson, et al., 1998; Vinson, et al., 1989). With that understanding, it is likely that the zone specific functions of the adrenal cells have related yet distinct mechanisms of signaling that require gap junction intercellular communication in the inner cortex and obviate it in outer cortex. Alternatively, the connexin 43 gap junctions in the adrenal cortex, as suggested in other systems, may be chemically rectified allowing the unidirectional flow of signals from the zona glomerulosa cells to the most peripheral cells in the zona fasciculata (Zahs, 1998). For example, the distribution of connexin 43 in the adrenal cortex could facilitate an asymmetric transfer of nutrients or signals that would promote tissue polarity in the adrenal gland similar to the role of gap junctions that has been suggested in the development of left-right patterning (Levin and Mercola, 1999; Meyer, et al., 1997). Pathologically, there is evidence that connexin 43 mutations are involved in the development of viscero-atrial heterotaxia, a condition which results in the malformation of the left- right axis in heart development and visceral organs in humans and murine models (Britz-Cunningham et al., 1995; Huang et al., 1998b). Therefore, distribution and expression of gap junctions appear to be integrally linked to function of numerous tissues.

The mechanism by which the adrenal gland maintains asymmetry of its zone specific functions across the cortex remains unclear. Therefore, to assess the degree to which connexin 43 expression was coincident with changes in steroidogenic enzyme expression, the P_{450} cytochrome enzymes specific to either the zona glomerulosa or fasciculata were evaluated. The changes in expression of the zona fasciculata specific enzyme, cytochrome P_{450} 11β hydroxylase were not informative in these studies because the monoclonal anti-11β hydroxylase antibody reacted nonspecifically in mouse tissues (data not shown). Nevertheless, glandular steroidogenisis as assessed by changes in cytochrome P_{450} aldosterone synthase expression and morphological changes in hypophysectomized mouse glands correlated with a reduction in connexin 43 abundance in the adrenal cortex. This again confirmed a difference between different functionally distinct compartments in the adrenal gland and gap junction expression. Future studies are necessary to determine if changes in gap junction rich zona fasciculata affect 11β hydroxylase expression in the inner cortex.

The connexin 43 deficient mouse glands demonstrated strikingly similar glandular morphology to that seen in adrenal glands from hypophysectomized mice, which are characterized by apparent glandular atrophy, a loss in lipid droplet storage and a reduction of zone specific morphology. In addition, the zona glomerulosa of the connexin 43 deficient mice lost much of their P_{450} aldosterone synthase expression, mimicking the findings in the hypophysectomized mouse adrenal glands. The difference in the developmental ages of the adult hypophysectomized mice and the neonatal connexin 43 knockout prevents a direct comparison of the two groups though zonation is established before birth in wildtype mice observed here and from other reports (Mitani, et al., 1999; Tajima, et al., 1999). Nevertheless, the comparative similarities in both the hypophysectomized and connexin 43 knockout adrenal glands are

significant for two reasons. First, the similarity in glandular features in both experimental groups suggests a need for proper connexin 43 expression in normal adrenal physiology. Secondly, the adrenal phenotype in the connexin 43 mice suggests a role for connexin 43 in the mouse adrenal gland independent of an ACTH derived stimulus, which may explain why hypophysectomy does not abolish all cortical gap junctions. However, the ACTH status of the connexin 43 deficient mice was not assessed. Therefore, it is potential that the pituitary gland itself in connexin 43 knockout mice may be compromised accounting for some of the observed defects in the null mouse. It is known, for example, that the anterior pituitary has abundant gap junctions and is communication competent (Gurerineau, et al., 1997; Morand et al., 1996; Wilfinger et al., 1984). Alternatively, connexin 43 expression in the prenatal mouse adrenal gland may be driven by unique factors independent of those in the adult mouse. Further studies of connexin expression in connexin 43 -/- mice would be informative in understanding the factors that influence adrenal morphology. Studies of cytogenesis and zonation in the rat adrenal gland would indicate the presence of different gap junction proteins in the adult versus fetal adrenal glands. In wildtype fetal adrenal glands, the zona fasciculata specific marker 11β hydroxylase positive cells are present throughout the entire adrenal cortex. Subsequent to the 11β hydroxylase expression, there is a sporadic occurrence of aldosterone synthase positive zona glomerulosa cells which later migrate and populate the outer adrenal zone seen postnatally in mature glands where zonation is fully established (Mitani, 1999; Wotus et al., 1998).

In the adrenal glands from the hypophysectomized mice, ACTH inputs alone may not fully explain the changes in gap junction expression and distribution. The effect of ACTH has been well documented in the adrenal gland. However, it is one of several peptides (including melanocortropin, melanocyte stimulating hormone and N-terminal pro-opiomelanocortin (N-

POMC) among others) that are generated from the cleavage of the propeptide pro-opiomelanocortin (POMC) synthesized in the pituitary gland (Bicknell, et al., 1996; James, 1992). Therefore, one must consider the possibility that these factors may potentially affect adrenal homeostasis and gap junction expression as well though their exact physiological role remains unclear (Pham-Huu-Trung, et al., 1982). Hypophysectomy effectively eliminates the possibility that the other pituitary derived proteins would influence these findings. Pharmacological agents such as the glucocorticoid analogues dexamethasone have been used to disrupt pituitary signaling and may prove useful to study gap junction expression in the adrenal gland as well (Guillemant, et al., 1982; Lesniewska, et al., 1992; Malendowicz, et al., 1992). But, the observation that the total loss of pituitary signals never completely abolishes adrenal connexin 43 expression in the innermost cortical cells supports the hypothesis that there may be additional factors that regulate cortical gap junction expression. Corticotropin releasing hormone from the hypothalamus, for example, has been postulated to act directly on the adrenal gland and reduce the atrophic features present in hypophysectomized animals circumventing its stimulation of the pituitary gland and ACTH release (Bornstein, et al., 1990). However, the fact that the injection of ACTH alone produces acute increases of gap junction expression in hypophysectomized mice supports the hypothesis that ACTH signaling plays a key role in promoting increased connexin 43 expression *in vivo*.

Future studies that address the direct effects of ACTH on adrenal gap junctions must be undertaken. The use of dexamethasone, which works via a negative feedback mechanism on the pituitary, is an alternative and reversible means to directly antagonize the ACTH stimulus to the adrenal gland (Roozendaal, et al., 1996). Conversely, the pharmalogical agent, metyrapone acts downstream of ACTH signaling by blocking P_{450} 11 β hydroxlyase activity in the zona

fasciculata (Fassnacht, et al., 1999). This method is another alternative to hypophysectomy that could be used to explore the degree to which ACTH affects gap junction expression. Ultimately a combined approach using agents in which both upstream and downstream pathways of ACTH signaling are inhibited is a potentially viable means to refine the effect of ACTH signaling on adrenal gap junction expression and distribution.

The observation that connexin 43 gap junction activity in the adrenal gland exhibit parallels communication pathways and differences in trophic state strongly suggest an inherent requirement for gap junction mediated intercellular communication in the adrenal gland. This finding has implications for cell-cell communication on a broader scale. It may be biologically more efficient for large populations of cells to function as a syncitium than as individual cells. Fauquier et al. demonstrate a neuron-like property in excitable non-endocrine folliculostellate cells of the anterior pituitary slices that could propagate calcium waves via resident gap junctions, thus facilitating large scale hormonal secretion (Fauquier et al., 2001). However, unlike the example described in the pituitary gland, the exploration of gap junctions in the intact adrenal gland is rare, and many of the factors that contribute to its coordinated glandular function have not been defined. A growing number of studies in transgenic mice studies in the testis, ovary and heart indicate that precise distribution of specific connexin isoforms is crucial for optimal tissue function (Juneja et al., 1999; Kidder and Mhawi, 2002; Lo, 2000; Lo et al., 1997; Severs et al., 2001).

6.3. Adrenal neoplasms

Neoplastic tissues may in some ways represent the most extreme cases of disruption of intercellular communication in the adrenal gland and other tissues. Indeed, that hypothesis is

corroborated by connexin 43 expression in the normal adrenals and neoplastic tissues. Although the connexin 43 profile had been described in lower mammals, it had never been characterized in humans before these studies (Murray et al., 2000). As is the case with adrenal glands from other animals, gap junctions in the adult human adrenal gland exhibited an identical distribution, being abundant in the zonae fasciculata/reticularis and nearly absent in the zona glomerulosa. Though connexin species 26, 32 (beta types) and connexin 43 (alpha type) were assayed, connexin 43 was the only connexin isoform identified in both the normal and neoplastic adrenal tissues observed.

The level of connexin abundance within the tissues was reflective of the distinct type of lesion. Specifically, the malignant adrenal carcinoma was most severe as the vast majority of connexin 43 was absent in these tissues when compared to normal human adrenal tissues. The benign adrenal adenomas maintained an intermediate level of connexin when compared to normal and carcinogenic adrenal tissues. Adenomatous tissues were also characterized by an aberrant pattern of connexin 43 staining suggestive of altered assembly and trafficking of connexin 43 to the plasma membrane (Johnson et al., 2002; Musil et al., 2000). The aberant connexin 43 staining is similar to that demonstrated by lysosomally targeted connexin 43 in other tumor cells though localization of mistrafficked gap junctions is cell type specific (Qin et al., 2001; Qin, et al., 2003). Conversely, tumorigenicity of cell expressing connexin 43 has been reduced in cells in which connexin 43 expression is increased (King et al., 2002).

Adrenal neoplasms exhibit a connexin phenotype that is reflective of their physiological state consistent to that demonstrated in the adrenal glands from hypophysectomized and connexin 43 null adrenal glands. This contention has been corroborated in studies in which a genetically normal epithelial cell line demonstrated modular gap junction expression that was

dependent on changes in nutrient availability and pH, which affected its ability to adopt a mitotic and apoptotic status. The authors proposed a scenario in which gap junctions act as a "physiological barometer" and thereby function as a biological gauge of tissue homeostasis (Wilson et al., 2000).

Normal adrenal tissues express specific levels of connexin in discrete patterns throughout the adrenal cortical zones. The native cellular distribution of connexin 43 in physiologically normal adrenal tissues is disrupted in neoplastic tissue. In cancerous lesions of the human adrenal glands examined, there was a strong correlation between the specific type of neoplastic tissue and connexin 43 abundance. This association may suggest that a specific level of connexin expression and, in turn, gap junction mediated intercellular communication may serve as a diagnostic hallmark of tumor grade in adrenal tissues. The pattern of adrenal expression of connexin 43 gap junctions also underscores the physiological differences of adrenal cortical tissues. Furthermore, there is evidence that points to the growth restrictive nature of gap junctions in both normal and neoplastic tissues. Since proliferation in a fundamental concern in neoplastic tissues, understanding the impact of gap junction expression in tumors may also serve to define the role of gap junctions in these tissues. An inverse correlation exists between proliferative rates and gap junction expression in primary bovine adrenal culutures and established murine cell lines (Decker et al., 1978; Murray and Shah, 1998). Mitotic rates in the normal adrenal cortex are also inversely proportional to connexin 43 gap junction expression (Murray et al., 1995). Evaluating the specific mitotic rates in the different adrenal lesions would complement the findings of gap junction expression differences in different lesions as adrenal adenoma are less aggressively dividing than adrenal carcinoma (Lucon, et al., 2002).

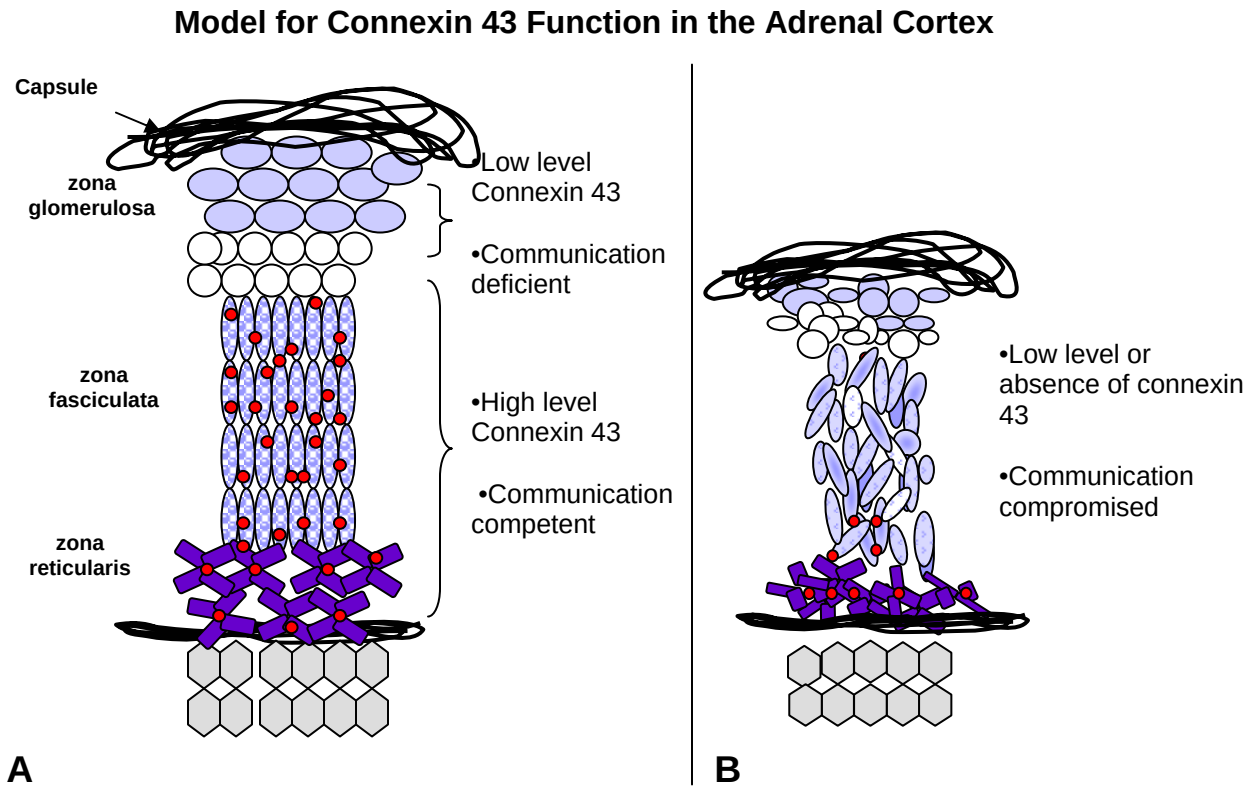

Figure 6-1 Gap Junctions and adrenal trophic state

Cortical gap junctions in the adrenal gland may act as mediators of adrenal trophic state. In normal adrenal cortex (A), proper distribution and abundance of connexin 43 gap junctions may facilitate the passage of the intercellular signals that promote proper expression of steroidogenic enzymes, lipid storage, and synchronous activity required for proper zonation and optimal adrenal homeostasis. Abrogation of normal adrenal function (B), may result in altered gap junction intercellular mediated communication and expression thereby promote altered glandular function, which could subsequently diminish overall adrenal integrity.

6.4. Summary

The differential expression of connexin 43 in the adrenal gland supports the presence of a gap junction mediated function *in vivo*. When adrenal homeostasis is disturbed via hypophysectomy, or neoplastic growth (humans), it results in a concomitant disruption of connexin 43 gap junctions in the adrenal cortex. This supports a biological requirement for gap junction mediated communication in endocrine function. Conversely, genomic ablation (mouse) of connexin 43 expression through transgenic means results in phenotypic changes in the adrenal gland. A model can be proposed in which adrenal homeostasis is related to functional communication between cells of the tissue (Fig. 6-1). Events that compromise cortical gap junctions in turn adversely affect its homeostasis and promote sub-optimal tissue function. The adrenal gap junctions are also functionally capable of discrete patterns of communication within the adrenal cortex. These findings establish, for the first time, that the intact adrenal gland maintains dynamic cell-cell coupling *in vivo* that is responsive to physiological demands. Refining the types of biologically relevant signals specific in to the function of connexin 43 gap junctions can now be addressed in terms of the zonal requirements of the gland. If adrenal gap junctions act as barometers of adrenal physiology then they must be responsive to the cascade of demands placed on the adrenal gland for growth, hormonal production and repair. This view of adrenal gap junction function can be applied to a gamut of tissues and organ systems in multicellular organisms, which must operate on a moment-to-moment basis to optimize a defined set of parameters necessary for their function. Questions regarding adrenal differentiation, zone specific hormone regulation and steroidogenesis can be asked with an appreciation that these processes are potentially supported and driven by cell-cell communication facilitated by gap junctions in the development of normal adrenal cell phenotypes or in cancer.

Future directions

The differences in zone specific expression of gap junctions raises new questions about their effect on cytogenesis and the development of different adrenal cellular subtypes. The H295 cell which have been used as a model of adrenal cell differentiation, express no gap junctions but are able to differentiate into cells exhibiting either a zona glomerulosa or zona fasciculata phenotype (Gazdar, et al., 1990). Introducing gap junctions into this cell line would elucidate the impact gap junctions have on the process P_{450} aldosterone synthase (zona glomerulosa specific) and 11β hydroxylase genes (zona fasciculata specific). Furthermore, there are questions that remain regarding the differential communication in the intact cortex. Using different gap junction permeable dyes in cultured adrenal sections would be benefical to understanding how this process changes as well. Organtypic cultures of adrenal glands would facilitate an understanding of how diverse processes such as zone specific hormonal response and apoptosis affect communication profiles in the primary adrenal tissues and, ultimately, enhance our understanding of the interdependence of gap junction function and adrenal physiology.

BIBLIOGRAPHY

Abraham, V., M.L. Chou, P. George, P. Pooler, A. Zaman, R.C. Savani, and M. Koval. 2001. Heterocellular gap junctional communication between alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 280:L1085-93.

Alexander, D.B., and G. Goldberg. 2003. Transfer of Biologically Important Molecules Between Cells Through Gap Junction Channels. *Curent Medicinal Chemistry*. 10:2045-2058.

Andreis, P.G., Rebuffat, P., Belloni, A.S., Neri, G., Cavallini, L., Gottardo, G., Mazzocchi, G., Coi, A., Malendowicz, L.K., Nussdorfer, G.G. 1989. Stereological and functional investigations on isolated adrenocortical cells: zona fasciculata/reticularis cells of chronically ACTH-treated rats. *Cell Tissue Res*. 258:43-51.

Ariyoshi, N., Kim, Y.C., Artemenko, I., Bhattacharyya, K.K., Jefcoate, C.R. 1998. Characterization of the rat Star gene that encodes the predominant 3.5-kilobase pair mRNA. ACTH stimulation of adrenal steroids in vivo precedes elevation of Star mRNA and protein. *J Biol Chem*. 273:7620-9.

Bauer, R., C. Lehmann, B. Fuss, F. Eckardt, and M. Hoch. 2002. The Drosophila gap junction channel gene innexin 2 controls foregut development in response to Wingless signalling. *J Cell Sci*. 115:1859-67.

Bevans, C.G., M. Kordel, S.K. Rhee, and A.L. Harris. 1998. Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. *J Biol Chem*. 273:2808-16.

Bicknell, A.B., Savva, D., Lowry, P.J. 1996. Pro-opiomelanocortin and adrenal function. *Endocr Res*. 22:385-93.

Bird, I.M., Mason, J. I., Rainey, W. E. 1998a. Battle of the kinases: integration of adrenal responses to cAMP, DG and Ca2+ at the level of steroidogenic cytochromes P450 and 3betaHSD expression in H295R cells. *Endocr Res*. 24:345-54.

Bird, I.M., Mason, J. I., Rainey, W. E. 1998b. Protein kinase A, protein kinase C, and Ca(2+)-regulated expression of 21-hydroxylase cytochrome P450 in H295R human adrenocortical cells. *J Clin Endocrinol Metab*. 83:1592-7.

Bland, M.L., Desclozeaux, M., Ingraham, H.A. 2003. Tissue growth and remodeling of the embryonic and adult adrenal gland. *Ann N Y Acad Sci*. 995:59-72.

Boger, D.L., J.E. Patterson, X. Guan, B.F. Cravatt, R.A. Lerner, and N.B. Gilula. 1998. Chemical requirements for inhibition of gap junction communication by the biologically active lipid oleamide. *Proc Natl Acad Sci U S A*. 95:4810-5.

Bornstein, S.R., Ehrhart, M., Scherbaum, W.A., Pfeiffer, E.F. 1990. Adrenocortical atrophy of hypophysectomized rats can be reduced by corticotropin-releasing hormone (CRH). *Cell Tissue Res*. 260:161-6.

Britz-Cunningham, S.H., M.M. Shah, C.W. Zuppan, and W.H. Fletcher. 1995. Mutations of the Connexin43 gap-junction gene in patients with heart malformations and defects of laterality. *N Engl J Med*. 332:1323-9.

Bruzzone, R., E.R. Trimble, A. Gjinovci, O. Traub, K. Willecke, and P. Meda. 1987. Regulation of pancreatic exocrine function: a role for cell-to-cell communication? *Pancreas*. 2:262-71.

Burghardt, R.C., and E. Anderson. 1979. Hormonal modulation of ovarian interstitial cells with particular reference to gap junctions. *J Cell Biol*. 81:104-14.

Calabrese, A., M. Guldenagel, A. Charollais, C. Mas, D. Caton, J. Bauquis, V. Serre-Beinier, D. Caille, G. Sohl, B. Teubner, S. Le Gurun, A. Trovato-Salinaro, D.F. Condorelli, J.A. Haefliger, K. Willecke, and P. Meda. 2001. Cx36 and the function of endocrine pancreas. *Cell Commun Adhes*. 8:387-91.

Cao, F., R. Eckert, C. Elfgang, J.M. Nitsche, S.A. Snyder, H.u. DF, K. Willecke, and B.J. Nicholson. 1998. A quantitative analysis of connexin-specific permeability differences of gap junctions expressed in HeLa transfectants and Xenopus oocytes. *J Cell Sci*. 111 (Pt 1):31-43.

Carsia, R.V., Nagele, R.G., Morita, Y., Tilly, K.I., Tilly, J.L. 1998. Models to elucidate the regulation of adrenal cell death. *Endocr Res*. 24:899-908.

Carsia, R.V.M.G.J., Gibney, J.A., Tilly, K.I., Tilly, J.L. 1996. Apoptotic cell death in the rat adrenal gland: an in vivo and in vitro investigation. *Cell Tissue Res*. 283:247-54.

Ceccatelli S, D.A., Villar MJ, Nicotera P. 1995. Adrenocortical apoptosis in hypophysectomized rats is selectively reduced by ACTH. *Neuroreport*. 6:342-4.

Chang, X., R. Patino, G. Yoshizaki, P. Thomas, and V.H. Lee. 2000. Hormonal regulation and cellular distribution of connexin 32.2 and connexin 32.7 RNAs in the ovary of Atlantic croaker. *Gen Comp Endocrinol*. 120:146-56.

Civitelli, R., K. Ziambaras, P.M. Warlow, F. Lecanda, T. Nelson, J. Harley, N. Atal, E.C. Beyer, and T.H. Steinberg. 1998. Regulation of connexin43 expression and function by prostaglandin E2 (PGE2) and parathyroid hormone (PTH) in osteoblastic cells. *J Cell Biochem*. 68:8-21.

Clark, A.J., Balla, T., Jones, M. R. Catt, K. J. 1992. Stimulation of early gene expression by angiotensin II in bovine adrenal glomerulosa cells: roles of calcium and protein kinase C. *Mol Endocrinol*. 6:1889-98.

Cook, D., and D. Loriaux. 1997. The incidental adrenal mass. *American Journal of Medicine*. 103:88-94.

Curtin, K.D., Z. Zhang, and R.J. Wyman. 1999. Drosophila has several genes for gap junction proteins. *Gene*. 232:191-201.

Davis, K.T., , Prentice, N., Gay, V. L., Murray, S. A. 2002. Gap junction proteins and cell-cell communication in the three functional zones of the adrenal gland. *Endocrinology*. 173:13-21.

Davis, K.T., I. McDuffie, L.A. Mawhinney, and S.A. Murray. 2000. Hypophysectomy results in a loss of connexin gap junction protein from the adrenal cortex. *Endocr Res*. 26:561-70.

De Feijter, A.W., J.S. Ray, C.M. Weghorst, J.E. Klaunig, J.I. Goodman, C.C. Chang, R.J. Ruch, and J.E. Trosko. 1990. Infection of rat liver epithelial cells with v-Ha-ras: correlation between oncogene expression, gap junctional communication, and tumorigenicity. *Mol Carcinog*. 3:54-67.

de Feijter-Rupp, H.L., T. Hayashi, G.H. Kalimi, P. Edwards, J.L. Redpath, C.C. Chang, E.J. Stanbridge, and J.E. Trosko. 1998. Restored gap junctional communication in non-tumorigenic HeLa-normal human fibroblast hybrids. *Carcinogenesis*. 19:747-54.

Decker, R.S. 1981. Gap junctions and steroidogenesis in the fetal mammalian adrenal cortex. *Dev Biol*. 82:20-31.

Decker, R.S., S.T. Donta, W.J. Larsen, and S.A. Murray. 1978. Gap junctions and ACTH sensitivity in Y-1 adrenal tumor cells. *J Supramol Struct*. 9:497-507.

Denner, K.R., W.E., Pezzi, V., Bird, I.M., Bernhardt, R., Mathis, J.M. 1996. Differentiated regulation of 11 beta hydroxylase and aldosterone synthase in human adrenocortical H295R cell. *Mol Cell Endocrinol*. 121:87-91.

Denoyelle, F., G. Lina-Granade, Plauchu H, R. Bruzzone, H. Chaib, F. Levi-Acobas, D. Weil, and C. Petit. 1998. Connexin-26 gene linked to a dominant deafness. *Nature*. 393:319-320.

DeoCampo, N.D., M.R. Wilson, and J.E. Trosko. 2000. Cooperation of bcl-2 and myc in the neoplastic transformation of normal rat liver epithelial cells is related to the down-regulation of gap junction-mediated intercellular communication. *Carcinogenesis*. 21:1501-6.

Diez, J.A., S. Ahmad, and W.H. Evans. 1999. Assembly of heteromeric connexons in guinea-pig liver en route to the Golgi apparatus, plasma membrane and gap junctions. *Eur J Biochem*. 262:142-8.

Duthe, F., E. Dupont, F. Verrecchia, I. Plaisance, N.J. Severs, D. Sarrouilhe, and J.C. Herve. 2000. Dephosphorylation agents depress gap junctional communication between rat cardiac cells without modifying the Connexin43 phosphorylation degree. *Gen Physiol Biophys*. 19:441-9.

Ehrenreich, H. 1999. The astrocytic endothelin system: toward solving a mystery focus on "distinct pharmacological properties of ET-1 and ET-3 on astroglial gap junctions and Ca(2+) signaling". *Am J Physiol*. 277:C614-5.

Ehrhart-Bornstein, M., Hinson, P., Bornstein, S. R. Scherbaum,W. A., Vinson, G. P. 1998. Intraadrenal Interactions in the Regulation of Adrenocortical Steroidogenesis. *Endocrine Reviews*. 19:101-43.

Eiberger, J., J. Degen, A. Romualdi, U. Deutsch, K. Willecke, and G. Sohl. 2001. Connexin genes in the mouse and human genome. *Cell Commun Adhes*. 8:163-5.

el-Fouly, M.H., J.E. Trosko, C.C. Chang, and S.T. Warren. 1989. Potential role of the human Ha-ras oncogene in the inhibition of gap junctional intercellular communication. *Mol Carcinog*. 2:131-5.

Engeland, W.C. 1999. Changes in the glomerulosa cell phenotype during adrenal regeneration in rats. *Am J Physiol*. 276:R1374-82.

Engeland, W.C., Gomez-Sanchez, C.E., Fitzgerald, D.A., Rogers, L.M., Holzwarth, M.A. 1996. Phenotypic changes and proliferation of adrenocortical cells during adrenal regeneration in rats. *Endocr Res*. 22:395-400.

Evans, W.H., and P.E. Martin. 2002. Gap junctions: structure and function (Review). *Mol Membr Biol*. 19:121-36.

Evert, M., T. Ott, A. Temme, K. Willecke, and F. Dombrowski. 2002. Morphology and morphometric investigation of hepatocellular preneoplastic lesions and neoplasms in connexin32-deficient mice. *Carcinogenesis*. 23:697-703.

Fassnacht, M., Hahner, S., Beuschlein, F., Klink, A., Reincke, M., Allolio, B. 1999. New mechanisms of adrenostatic compounds in a human adrenocortical cancer cell line. *Eur J Clin Invest*. 30:76-82.

Fauquier, T., N.C. Guerineau, R.A. McKinney, K. Bauer, and P. Mollard. 2001. Folliculostellate cell network: a route for long-distance communication in the anterior pituitary. *Proc Natl Acad Sci U S A*. 98:8891-6.

Francis, D., K. Stergiopoulos, J.F. Ek-Vitorin, F.L. Cao, S.M. Taffet, and M. Delmar. 1999. Connexin diversity and gap junction regulation by pHi. *Dev Genet*. 24:123-36.

Friend, D.S., and N.B. Gilula. 1972. Variations in tight and gap junctions in mammalian tissues. *J Cell Biol*. 53.

Gallo-Payet, N., and M. Payet. 2003. Mechanism of ACTH:beyond cAMP. *Microsc Res Tech*. 61:275-87.

Gazdar, A.F., Oie, H.K., Shackleton, C.H., Chen, T.R., Triche, T.J., Myers, C.E., Chrousos, G.P., Brennan, M.F., Stein, C.A., La Rocca, R.V. 1990. Establishment and characterization of a human adrenocortical carcinoma cell line that expresses multiple pathways of steroid biosynthesis. *Cancer Res*. 50:5488-96.

Goldberg, G.S., P.D. Lampe, D. Sheedy, C.C. Stewart, B.J. Nicholson, and C.C. Naus. 1998. Direct isolation and analysis of endogenous transjunctional ADP from Cx43 transfected C6 glioma cells. *Exp Cell Res*. 239:82-92.

Goldberg, G.S., A.P. Moreno, and P.D. Lampe. 2002. Gap junctions between cells expressing connexin 43 or 32 show inverse permselectivity to adenosine and ATP. *J Biol Chem*. 277:36725-30.

Green, L.M., D.K. Murray, D.T. Tran, G.A. Nelson, M.M. Shah, and R.A. Luben. 2001. A spontaneously arising mutation in connexin32 with repeated passage of FRTL-5 cells coincides with increased growth rate and reduced thyroxine release. *J Mol Endocrinol*. 27:145-63.

Guan, X., B.F. Cravatt, G.R. Ehring, J.E. Hall, D.L. Boger, R.A. Lerner, and N.B. Gilula. 1997. The sleep-inducing lipid oleamide deconvolutes gap junction communication and calcium wave transmission in glial cells. *J Cell Biol*. 139:1785-92.

Guerrier, A., P. Fonlupt, I. Morand, R. Rabilloud, C. Audebet, V. Krutovskikh, D. Gros, B. Rousset, and Y. Munari-Silem. 1995. Gap junctions and cell polarity: connexin32 and connexin43 expressed in polarized thyroid epithelial cells assemble into separate gap junctions, which are located in distinct regions of the lateral plasma membrane domain. *J Cell Sci*. 108 (Pt 7):2609-17.

Guillemant, J., Guillemant, S. 1982. Effect of dexamethasone on steroidogenesis and on adrenocortical cyclic AMP and cyclic GMP responses to ACTH. *Horm Metab Res*. 14:547-50.

Gurerineau, N.C., McKinney, R.A., Debanne, D., Mollard, P. Gahwiler, B.H. 1997. Organotypic cultures of the rat anterior pituitary: morphology, physiology and cell-to-cell communication. *J Neurosci Methods*. 73:169-176.

Hagendorff, A., S. Kirchhoff, O. Kruger, D. Eckhardt, A. Plum, B. Schumacher, and C. Wolpert. 2001. [Electrophysiological characterization of connexin 40 deficient hearts--in vivo studies in mice]. *Z Kardiol*. 90:898-905.

Halban, P.A., C.B. Wollheim, B. Blondel, P. Meda, E.N. Niesor, and D.H. Mintz. 1982. The possible importance of contact between pancreatic islet cells for the control of insulin release. *Endocrinology*. 111:86-94.

Harris, A.L. 2001. Emerging issues of connexin channels: biophysics fills the gap. *Q Rev Biophys*. 34:325-472.

He, D.S., J.X. Jiang, S.M. Taffet, and J.M. Burt. 1999. Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells. *Proc Natl Acad Sci U S A*. 96:6495-500.

Holder, J.W., E. Elmore, and J.C. Barrett. 1993. Gap junction function and cancer. *Cancer Res*. 53:3475-85.

Holmes, M.C., Kotelevtsev, Y., Mullins, J. J., Seckl, J. R. 2001. Phenotypic analysis of mice bearing targeted deletions of 11beta-hydroxysteroid dehydrogenases 1 and 2 genes. *Mol Cell Endocrinol*. 171:15-20.

Hough, A.J., Hollifield, J. W., Page, D.L., Hartmann, W. H. 1979. Prognostic factors in adrenal cortical tumors. A mathematical analysis of clinical and morphologic data. *Am J Clin Pathol*. 72:390-9.

Huang, G.Y., E.S. Cooper, K. Waldo, M.L. Kirby, N.B. Gilula, and C.W. Lo. 1998a. Gap junction-mediated cell-cell communication modulates mouse neural crest migration. *J Cell Biol*. 143:1725-34.

Huang, G.Y., A. Wessels, B.R. Smith, K.K. Linask, J.L. Ewart, and C.W. Lo. 1998b. Alteration in connexin 43 gap junction gene dosage impairs conotruncal heart development. *Dev Biol*. 198:32-44.

Hyatt, P.J., Wale, L. W., Bell, J. B., Tait, J. F., Tait, S. A. 1980. Cyclic AMP levels in purified rat adrenal zonae fasciculata and reticularis cells and the effect of adrenocorticotrophic hormone. *J Endocrinol*. 85:435-42.

James, V.H.T. 1992. The Adrenal Gland. *In* Comprehensive Endocrinology. L. Martini, editor. Raven, New York. 513.

Johnson, R.G., R.A. Meyer, X.R. Li, D.M. Preus, L. Tan, H. Grunenwald, A.F. Paulson, D.W. Laird, and J.D. Sheridan. 2002. Gap junctions assemble in the presence of cytoskeletal inhibitors, but enhanced assembly requires microtubules. *Exp Cell Res*. 275:67-80.

Juneja, S.C., K.J. Barr, G.C. Enders, and G.M. Kidder. 1999. Defects in the germ line and gonads of mice lacking connexin43. *Biol Reprod*. 60:1263-70.

Kavanagh, T.J., G. Raghu, C.C. White, G.M. Martin, P.S. Rabinovitch, and D.L. Eaton. 1994. Enhancement of glutathione content in glutathione synthetase-deficient fibroblasts from a patient with 5-oxoprolinuria via metabolic cooperation with normal fibroblasts. *Exp Cell Res*. 212:69-76.

Kidder, G.M., and A.A. Mhawi. 2002. Gap junctions and ovarian folliculogenesis. *Reproduction*. 123:613-20.

King, T.J., L.H. Fukushima, Y. Yasui, P.D. Lampe, and J.S. Bertram. 2002. Inducible expression of the gap junction protein connexin43 decreases the neoplastic potential of HT-1080 human fibrosarcoma cells in vitro and in vivo. *Mol Carcinog*. 35:29-41.

Krutovskikh, V.A., C. Piccoli, and H. Yamasaki. 2002. Gap junction intercellular communication propagates cell death in cancerous cells. *Oncogene*. 21:1989-99.

Kumar, N.M., and N.B. Gilula. 1996. The gap junction communication channel. *Cell*. 84:381-8.

Laing, J.G., P.N. Tadros, E.M. Westphale, and E.C. Beyer. 1997. Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. *Exp Cell Res*. 236:482-92.

Laird, D.W., K.L. Puranam, and J.P. Revel. 1991. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem J*. 273(Pt 1):67-72.

Lampe, P.D., and A.F. Lau. 2000. Regulation of gap junctions by phosphorylation of connexins. *Arch Biochem Biophys*. 384:205-15.

Lampe, P.D., E.M. TenBroek, J.M. Burt, W.E. Kurata, R.G. Johnson, and A.F. Lau. 2000. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol*. 149:1503-12.

Landesman, Y., T.W. White, T.A. Starich, J.E. Shaw, D.A. Goodenough, and D.L. Paul. 1999. Innexin-3 forms connexin-like intercellular channels. *J Cell Sci*. 112 (Pt 14):2391-6.

Lehoux, J.G., Bird, I. M., Briere, N., Martel, D., Ducharme, L. 1997. Influence of dietary sodium restriction on angiotensin II receptors in rat adrenals. *Endocrinology*. 138:5238-47.

Lesniewska, B., Nowak, K. W., Malendowicz, L. K. 1992. Dexamethasone-induced adrenal cortex atrophy and recovery of the gland from partial, steroid-induced atrophy. *Experimental Clinical Endocrinology*. 100:133-9.

Levin, M., and M. Mercola. 1999. Gap junction-mediated transfer of left-right patterning signals in the early chick blastoderm is upstream of Shh asymmetry in the node. *Development*. 126:4703-14.

Li, W.E., and J.I. Nagy. 2000. Connexin43 phosphorylation state and intercellular communication in cultured astrocytes following hypoxia and protein phosphatase inhibition. *Eur J Neurosci*. 12:2644-50.

Liao, Y., K.H. Day, D.N. Damon, and B.R. Duling. 2001. Endothelial cell-specific knockout of connexin 43 causes hypotension and bradycardia in mice. *Proc Natl Acad Sci U S A*. 98:9989-94.

Liaskos, P., E. Chambaz, J. Feige, and G. De Faye. 1998. Expression of ACTH receptors (MC2-R) and (MC5-R) in glomerulosa and the fasciculata-reticularis zones of bovine adrenal cortex. *Endocrine Res*. 24:427-32.

Lo, C.W. 2000. Role of gap junctions in cardiac conduction and development: insights from the connexin knockout mice. *Circ Res*. 87:346-8.

Lo, C.W., M.F. Cohen, G.Y. Huang, B.O. Lazatin, N. Patel, R. Sullivan, C. Pauken, and S.M. Park. 1997. Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. *Dev Genet*. 20:119-32.

Lucon, A.M., Pereira, M.A., Mendonca, B.B., Zerbini, M.C., Saldanha, L.B., Arap S. 2002. Adrenocortical tumors: results of treatment and study of Weiss's score as a prognostic factor. *Rev Hosp Clin Fac Med Sao Paulo*. 57:251-6.

Malendowicz, L.K., Nussdorfer, G. G., Markowska, A., Nowak, K. W. 1992. Analysis of the preventive action of ACTH on dexamethasone-induced adrenocortical atrophy in the rat. *Cytobios*. 71:191-9.

Marx, C., Wolkersdorfer, G. W., Brown, J. W., Scherbaum, W.A., Bornstein, S. R. 1996. MHC class II expression--a new tool to assess dignity in adrenocortical tumours. *J Clin Endocrinol Metab*. 81:4488-4491.

Mazzocchi, G., Malendowicz, L. K., Rebuffat, P., Robba, C., Gottardo, G., Nussdorfer, G. G. 1986. Short- and long-term effects of ACTH on the adrenal zona glomerulosa of the rat. A coupled stereological and enzymological study. *Cell Tissue Res*. 243:303-10.

Meda, P. 1996a. Gap junction involvement in secretion: the pancreas experience. *Clin Exp Pharmacol Physiol*. 23:1053-7.

Meda, P. 1996b. The role of gap junction membrane channels in secretion and hormonal action. *J Bioenerg Biomembr*. 28:369-77.

Meda, P. 2000. Probing the function of connexin channels in primary tissues. *Methods*. 20:232-44.

Meda, P., R. Bruzzone, M. Chanson, D. Bosco, and L. Orci. 1987. Gap junctional coupling modulates secretion of exocrine pancreas. *Proc Natl Acad Sci U S A*. 84:4901-4.

Meda, P., M. Chanson, M. Pepper, E. Giordano, D. Bosco, O. Traub, K. Willecke, A. el Aoumari, D. Gros, E.C. Beyer, and et al. 1991. In vivo modulation of connexin 43 gene expression and junctional coupling of pancreatic B-cells. *Exp Cell Res*. 192:469-80.

Meda, P., R.L. Michaels, P.A. Halban, L. Orci, and J.D. Sheridan. 1983. In vivo modulation of gap junctions and dye coupling between B-cells of the intact pancreatic islet. *Diabetes*. 32:858-68.

Meda, P., M.S. Pepper, O. Traub, K. Willecke, D. Gros, E. Beyer, B. Nicholson, D. Paul, and L. Orci. 1993. Differential expression of gap junction connexins in endocrine and exocrine glands. *Endocrinology*. 133:2371-8.

Mehta, P.P., J.S. Bertram, and W.R. Loewenstein. 1986. Growth inhibition of transformed cells correlates with their junctional communication with normal cells. *Cell*. 44:187-96.

Mesnil, M., M. Asamoto, C. Piccoli, and H. Yamasaki. 1994. Possible molecular mechanism of loss of homologous and heterologous gap junctional intercellular communication in rat liver epithelial cell lines. *Cell Adhes Commun*. 2:377-84.

Meyer, R.A., Cohen, M. F., Recalde, S., Zakany, J., Bell, S. M., Scott, W. J., Jr., Lo, C. W. 1997. Developmental regulation and asymmetric expression of the gene encoding Cx43 gap junctions in the mouse limb bud. *Dev Genet*. 21:290-300.

Migally, N. 1980. Effect of cyproterone acetate on structure of adrenal cortex in hypophysectomized, ACTH treated mice. *Arch Androl*. 4:109-113.

Mitani, F., Mukai, K., Miyamoto, H., Suematsu, M., Ishimura, Y. 1999. Development of functional zonation in the rat adrenal cortex. *Endocrinology*. 140:3342-53.

Mitani, F., Mukai, K., Miyamoto, H., Suematsu, M., Ishimura, Y. 2003. The undifferentiated cell zone is a stem cell zone in adult rat adrenal cortex. *Biochim Biophys Acta*. 619:317-24.

Mitani, F., Suzuki, H., Hata, J., Ogishima, T., Shimada, H., Ishimura, Y. 1994. A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology*. 135:431-8.

Morand, I., P. Fonlupt, A. Guerrier, J. Trouillas, A. Calle, C. Remy, B. Rousset, and Y. Munari-Silem. 1996. Cell-to-cell communication in the anterior pituitary: evidence for gap junction-mediated exchanges between endocrine cells and folliculostellate cells. *Endocrinology*. 137:3356-67.

Mukai, K., Nagasawa, H., Agake-Suzuki, R., Mitani, F., Totani, K., Yanai, N., Obinata, M., Suematsu, M., Ishimura, Y. 2002. Conditionally immortalized adrenocortical cell lines. *Eur J Biochem*. 269:69-81.

Munari-Silem, Y., M.C. Lebrethon, I. Morand, B. Rousset, and J.M. Saez. 1995. Gap junction-mediated cell-to-cell communication in bovine and human adrenal cells. A process whereby cells increase their responsiveness to physiological corticotropin concentrations. *J Clin Invest*. 95:1429-39.

Murray, S.A., K. Davis, L.M. Fishman, and S.R. Bornstein. 2000. Alpha1 connexin 43 gap junctions are decreased in human adrenocortical tumors. *J Clin Endocrinol Metab*. 85:890-5.

Murray, S.A., and W.H. Fletcher. 1984. Hormone-induced intercellular signal transfer dissociates cyclic AMP-dependent protein kinase. *J Cell Biol*. 98:1710-9.

Murray, S.A., U.A. Oyoyo, S.Y. Pharrams, N.M. Kumar, and N.B. Gilula. 1995. Characterization of gap junction expression in the adrenal gland. *Endocr Res*. 21:221-9.

Murray, S.A., Pharrams, S.Y. 1997. Comparison of gap junction expression in the adrenal gland. *Microsc Res Tech*. 36:510-9.

Murray, S.A., and U.S. Shah. 1998. Modulation of adrenal gap junction expression. *Horm Metab Res*. 30:426-31.

Musil, L.S., and D.A. Goodenough. 1993. Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell*. 74:1065-77.

Musil, L.S., A.C. Le, J.K. VanSlyke, and L.M. Roberts. 2000. Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J Biol Chem*. 275:25207-15.

Naus, C.C., S.L. Bond, J.F. Bechberger, and W. Rushlow. 2000. Identification of genes differentially expressed in C6 glioma cells transfected with connexin43. *Brain Res Brain Res Rev*. 32:259-66.

Nicholson, S.M., and R. Bruzzone. 1997. Gap junctions: getting the message through. *Curr Biol*. 7:R340-4.

Nickerson, P.A., Brownie, A.C. 1987. Effect of hypophysectomy on the volume and ultrastructure of zona glomerulosa in rat adrenal. *Endocrinol Exp*. 9:187-196.

Ogishima, T., H. Suzuki, J. Hata, Mitani F, and Y. Ishimura. 1992. Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-450 11β in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology*. 130:2971-2977.

Oyoyo, U.A., U.S. Shah, and S.A. Murray. 1997. The role of alpha1 (connexin-43) gap junction expression in adrenal cortical cell function. *Endocrinology*. 138:5385-97.

Parmer, R.J., Zinder, O. 2002. Catecholaminergic pathways, chromaffin cells, and human disease. *Ann N Y Acad Sci*. 971:497-505.

Pham-Huu-Trung, M.T., De Smitter, N., Bogio, A., Bertagna, X., Girard, F. 1982. Responses of isolated guinea-pig adrenal cells to ACTH and pro-opiocortin-derived peptides. *Endocrinology*. 110:1819-21.

Phelan, P., and T.A. Starich. 2001. Innexins get into the gap. *Bioessays*. 23:388-96.

Plum, A., Hallas, G. Magin, T., Dombrowski, F., Hagendorff, A., Schumacher, B., Wolpert, C., Kim, J., Lamers, W. H., Evert, M., Meda, P. Traub, O., Willecke, K. 2000. Unique and shared functions of different connexins in mice. *Curr Biol*. 10:1083-91.

Qin, H., Q. Shao, D.J. Belliveau, and D.W. Laird. 2001. Aggregated DsRed-tagged Cx43 and over-expressed Cx43 are targeted to lysosomes in human breast cancer cells. *Cell Commun Adhes*. 8:433-9.

Qin, H., Shao, Q., Igdoura, S. A., Alaoui-Jamali, M.A., Laird, D.W. 2003. Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. *J Biol Chem*. 278:30005-14.

Quinn, S.J., and G.H. Williams. 1992. Regulatiion of Aldosterone Secretion. *In* Comprehensive Endocrinology: The Adrenal Gland. L. Martini, editor. Raven Press, New York. 156-189.

Rainey, W.E. 1999. Adrenal zonation: clues from 11beta-hydroxylase and aldosterone synthase. *Mol Cell Endocrinol*. 151:151-60.

Rainey, W.E., Carr, B.R., Sasano, H., Suzuki, T., Mason, J.I. 2002. Dissecting human adrenal androgen production. *Trends Endocrinol Metab*. 13:234-9.

Raza, F.S., Puddefoot, J.R., Vinson, G.P. 1998. Pref-1, SF-1 and adrenocortical zonation. *Endocr Res*. 24:977-81.

Reaume, A.G., P.A. de Sousa, S. Kulkarni, B.L. Langille, D. Zhu, T.C. Davies, S.C. Juneja, G.M. Kidder, and J. Rossant. 1995. Cardiac malformation in neonatal mice lacking connexin43. *Science*. 267:1831-4.

Rebuffat, P., Neri, G., Andreis, P. G., Belloni, A. S., Mazzocchi, G., Musajo, F.G., Nussdorfer, G. G. 1991. Effects of prolonged treatment with adrenocorticotropin on the morphology and function of rat adrenocortical autotransplants. *Cytobios*. 65:101-13.

Risek, B., F.G. Klier, and N.B. Gilula. 1994. Developmental regulation and structural organization of connexins in epidermal gap junctions. *Dev Biol*. 164:183-96.

Rivedal, E., H. Yamasaki, and T. Sanner. 1994. Inhibition of gap junctional intercellular communication in Syrian hamster embryo cells by TPA, retinoic acid and DDT. *Carcinogenesis*. 15:689-94.

Robinson, P.M., Comline, R.S., Fowden, A.L., Silver, M. 1983. Adrenal cortex of fetal lamb: changes after hypophysectomy and effects of Synacthen on cytoarchitecture and secretory activity. *Q J Exp Physiol*. 68:15-27.

Romanello, M., L. Moro, D. Pirulli, S. Crovella, and P. D'Andrea. 2001. Effects of cAMP on intercellular coupling and osteoblast differentiation. *Biochem Biophys Res Commun*. 282:1138-44.

Roozendaal, B., Carmi, O., McGaugh, J.L. 1996. Adrenocortical suppression blocks the memory-enhancing effects of amphetamine and epinephrine. *Proc Natl Acad Sci U S A*. 93:1429-33.

Roscoe, W.A., K.J. Barr, A.A. Mhawi, D.K. Pomerantz, and G.M. Kidder. 2001. Failure of spermatogenesis in mice lacking connexin43. *Biol Reprod*. 65:829-38.

Saez, J.C., J.A. Connor, D.C. Spray, and M.V. Bennett. 1989a. Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. *Proc Natl Acad Sci U S A*. 86:2708-12.

Saez, J.C., W.A. Gregory, T. Watanabe, R. Dermietzel, E.L. Hertzberg, L. Reid, M.V. Bennett, and D.C. Spray. 1989b. cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. *Am J Physiol*. 257:C1-11.

Saffitz, J.E., J.G. Laing, and K.A. Yamada. 2000. Connexin expression and turnover : implications for cardiac excitability. *Circ Res*. 86:723-8.

Schiller, P.C., P.P. Mehta, B.A. Roos, and G.A. Howard. 1992. Hormonal regulation of intercellular communication: parathyroid hormone increases connexin 43 gene expression and gap-junctional communication in osteoblastic cells. *Mol Endocrinol*. 6:1433-40.

Serre-Beinier, V., C. Mas, A. Calabrese, D. Caton, J. Bauquis, D. Caille, A. Charollais, V. Cirulli, and P. Meda. 2002. Connexins and secretion. *Biol Cell*. 94:477-92.

Severs, N.J. 1999. Cardiovascular disease. *Novartis Found Symp*. 219:188-206; discussion 206-11.

Severs, N.J. 2000. The cardiac muscle cell. *Bioessays*. 22:188-99.

Severs, N.J., S. Rothery, E. Dupont, S.R. Coppen, H.I. Yeh, Y.S. Ko, T. Matsushita, R. Kaba, and D. Halliday. 2001. Immunocytochemical analysis of connexin expression in the healthy and diseased cardiovascular system. *Microsc Res Tech*. 52:301-22.

Sewer, M., and M. Waterman. 2003. ACTH modulation of transcription factors responsible for steroid hydroxylase gene expression in the adrenal cortex. *Microsc Res Tech*. 61:300-7.

Shah, U.S., and S.A. Murray. 2001. Bimodal inhibition of connexin 43 gap junctions decreases ACTH-induced steroidogenesis and increases bovine adrenal cell population growth. *J Endocrinol*. 171:199-208.

Stock, A., Sies, H., Stahl, W. 1998. Enhancement of gap junctional communication and connexin43 expression by thyroid hormones. *Biochem Pharmacol*. 55:475-9.

Stumpel, F., T. Ott, K. Willecke, and K. Jungermann. 1998. Connexin 32 gap junctions enhance stimulation of glucose output by glucagon and noradrenaline in mouse liver. *Hepatology*. 28:1616-20.

Tajima, T., Ma, X.M., Bornstein S.R., Aguilera, G. 1999. Prenatal dexamethasone treatment does not prevent alterations of the hypothalamic pituitary adrenal axis in steroid 21-hydroxylase deficient mice. *Endocrinology*. 140:3354-62.

Todman, M.G., R.A. Baines, L.A. Stebbings, J.A. Davies, and J.P. Bacon. 1999. Gap-Junctional communication between developing Drosophila muscles is essential for their normal development. *Dev Genet*. 24:57-68.

Tremblay, A., Parker, K.L., LeHoux, J.G. 1992. Dietary potassium supplementation and sodium restriction stimulate aldosterone synthase but not 11ß-hydroxylase P-450 messenger ribonucleic acid accumulation in rat adrenals and require angiotensin II production. *Endocrinology*. 130:3152-3158.

Trosko, J.E., C.C. Chang, B.V. Madhukar, and J.E. Klaunig. 1990. Chemical, oncogene and growth factor inhibition gap junctional intercellular communication: an integrative hypothesis of carcinogenesis. *Pathobiology*. 58:265-78.

Trosko, J.E., C.C. Chang, B. Upham, and M. Wilson. 1998. Epigenetic toxicology as toxicant-induced changes in intracellular signalling leading to altered gap junctional intercellular communication. *Toxicol Lett*. 102-103:71-8.

Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999a. Electron cryo-crystallography of a recombinant cardiac gap junction channel. *Novartis Found Symp*. 219:22-30; discussion 31-43.

Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999b. Expression, two-dimensional crystallization, and electron cryo-crystallography of recombinant gap junction membrane channels. *J Struct Biol*. 128:98-105.

van der Heyden, M.A., J.M. Veltmaat, J.A. Hendriks, O.H. Destree, and L.H. Defize. 2000. Dynamic connexin43 expression and gap junctional communication during endoderm differentiation of F9 embryonal carcinoma cells. *Eur J Cell Biol*. 79:272-82.

van Slooten, H., Schaberg, A., Smeenk, D., Moolenaar, A. J. 1985. Morphologic characteristics of benign and malignant adrenocortical tumors. *Cancer*. 55:766-773.

VanSlyke, J.K., and L.S. Musil. 2000. Analysis of connexin intracellular transport and assembly. *Methods*. 20:156-64.

Veenstra, R.D. 1990. Voltage-dependent gating of gap junction channels in embryonic chick ventricular cell pairs. *Am J Physiol*. 258:C662-72.

Veenstra, R.D. 1996. Size and selectivity of gap junction channels formed from different connexins. *J Bioenerg Biomembr*. 28:327-37.

Veenstra, R.D., H.Z. Wang, D.A. Beblo, M.G. Chilton, A.L. Harris, E.C. Beyer, and P.R. Brink. 1995. Selectivity of connexin-specific gap junctions does not correlate with channel conductance. *Circ Res*. 77:1156-65.

Vinson, G.P., Ho, M. M., Puddefoot, J. R. 1998. Adrenocortical zonation and the adrenal renin-angiotensin system. *Endocr Res*. 24:677-86.

Vinson, G.P., Laird, S.M., Whitehouse, B.J., Hinson, J.P. 1989. Specific effects of agonists of the calcium messenger system on secretion of 'late-pathway' steroid products by intact tissue and dispersed cells of the rat adrenal zona glomerulosa. *J Mol Endocrinol*. 2:157-62.

Vozzi, C., S. Ullrich, A. Charollais, J. Philippe, L. Orci, and P. Meda. 1995. Adequate connexin-mediated coupling is required for proper insulin production. *J Cell Biol*. 131:1561-72.
Vultur, A., E. Tomai, K. Peebles, A.M. Malkinson, N. Grammatikakis, P.G. Forkert, and L. Raptis. 2003. Gap junctional intercellular communication in cells isolated from urethane-induced tumors in A/J mice. *DNA Cell Biol*. 22:33-40.
Wang, Y., and B. Rose. 1995. Clustering of Cx43 cell-to-cell channels into gap junction plaques: regulation by cAMP and microfilaments. *J Cell Sci*. 108 (Pt 11):3501-8.
Weiss, L.M. 1984. Comparative histologic study of 43 metastasizing adrenocortical tumours. *Am J Surg Pathol*. 8:163-169.
White, T.W., R. Bruzzone, S. Wolfram, D.L. Paul, and D.A. Goodenough. 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*. 125:879-92.
White, T.W., M.R. Deans, D.P. Kelsell, and D.L. Paul. 1998. Connexin mutations in deafness. *Nature*. 394:630-1.
Wilfinger, W.W., W.J. Larsen, T.R. Downs, and D.L. Wilbur. 1984. An in vitro model for studies of intercellular communication in cultured rat anterior pituitary cells. *Tissue Cell*. 16:483-97.
Willecke, K., J. Eiberger, J. Degen, D. Eckardt, A. Romualdi, M. Guldenagel, U. Deutsch, and G. Sohl. 2002. Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem*. 383:725-37.
Wilson, M.R., T.W. Close, and J.E. Trosko. 2000. Cell population dynamics (apoptosis, mitosis, and cell-cell communication) during disruption of homeostasis. *Exp Cell Res*. 254:257-68.
Wotus, C., B.K. Levay-Young, L.M. Rogers, C.E. Gomez-Sanchez, and W.C. Engeland. 1998. Development of adrenal zonation in fetal rats defined by expression of aldosterone synthase and 11beta-hydroxylase. *Endocrinology*. 139:4397-403.
Xia, Y., and J.E. Wikberg. 1996. Localization of ACTH receptor mRNA by in situ hybridization in mouse adrenal gland. *Cell Tissue Res*. 286:63-8.
Yamasaki, H. 1995. Non-genotoxic mechanisms of carcinogenesis: studies of cell transformation and gap junctional intercellular communication. *Toxicol Lett*. 77:55-61.
Yeager, M. 1998. Structure of cardiac gap junction intercellular channels. *J Struct Biol*. 121:231-45.
Yeager, M., V.M. Unger, and M.M. Falk. 1998. Synthesis, assembly and structure of gap junction intercellular channels. *Curr Opin Struct Biol*. 8:517-24.
Yum, S.W., K.A. Kleopa, S. Shumas, and S.S. Scherer. 2002. Diverse trafficking abnormalities of connexin32 mutants causing CMTX. *Neurobiol Dis*. 11:43-52.
Zahs, K.R. 1998. Heterotypic coupling between glial cells of the mammalian central nervous system. *Glia*. 24:85-96.
Zelante, L., P. Gasparini, X. Estivill, S. Melchionda, L. D'Agruma, N. Govea, M. Mila, M.D. Monica, J. Lutfi, M. Shohat, E. Mansfield, K. Delgrosso, E. Rappaport, S. Surrey, and P. Fortina. 1997. Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. *Hum Mol Genet*. 6:1605-9.