NON-CLASSICAL GLUCOCORTICOID RECEPTOR ACTION REGULATES GAP JUNCTION INTERCELLULAR COMMUNICATION AND NEURAL PROGENITOR CELL PROLIFERATION

by

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Glucocorticoids (GCs) are administered to neonates and to pregnant mothers for the treatment of complications arising from premature birth and for congenital adrenal hyperplasia; however, antenatal exposure to GCs may trigger adverse neurological side effects due in part to reduced neural progenitor cell (NPC) proliferation. While many established cell cycle regulators impact NPC proliferation, other molecules also influence proliferation. An example is the gap junction protein connexin 43 (Cx43), although its precise role and mechanism of regulation remain unresolved. Gap junction intercellular communication (GJIC) is influenced by GCs in some cells, but such hormone effects and resulting functional consequences have not been examined in coupled stem cells. We found that both continuous and transient exposure of embryonic (E14.5) mouse neurosphere cultures to the glucocorticoid dexamethasone (DEX) limits proliferation of coupled NPCs, which is manifested by both a reduction in S phase progression and enhanced cell cycle exit. A short (i.e. 1hr) DEX treatment also reduced GJIC as measured by live cell fluorescence recovery after photobleaching (FRAP). GC effects on GJIC in NPCs are transcription-independent and mediated through plasma membrane glucocorticoid receptors (GRs). This non-classical pathway appears to operate through lipid-raft associated GRs through a site-specific, MAPK-dependent phosphorylation of Cx43, which is linked to GR via caveolin-1

and c-src. These effects were absent in caveolin-1 knockout NPCs indicating that caveolin-1 is an essential component for this signaling pathway. As transient pharmacologic inhibition of GJIC triggers reduced S phase progression but not enhanced cell cycle exit, the non-classical GR signaling pathway may operate via distinct downstream effectors to alter the proliferative capacity of NPCs.

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PREFACE

There are a number of individuals that have supported me during my graduate studies and made this thesis possible. I have had the good fortune of close friends that encouraged and helped me throughout this process. Vanessa, my running buddy, friend, and thesis editor, thank you for all of your help and for helping to preserve my sanity. Melanie, my sidekick in the laboratory, four years would have been much more difficult without your company and friendship. Maxx and Danny, nerd herd meetings, your friendship, and your camaraderie since the beginning of medical school have helped sustain this effort. Thank you so much!

I also cannot overstate the benefit I've gained from having fantastic mentors and a committed thesis committee. Dr. Keiko Ozato took me into her laboratory at the NIH eight years ago and inculcated in me an appreciation and love of science. Keiko's mentorship and guidance, more than any other single individual, inspired me to pursue this career.

My committee members Drs. Berman, Monaghan, Romero, and Chu provided significant insights and guidance as I developed this project. Dr. Romero has been particularly invaluable in spending countless hours providing technical advice and expertise for a number of key experiments. Finally, Dr. Aizenman, my committee chair has been especially important to my progress, not only by helping to guide my project but also by constantly pushing me to be a better, more rigorous, scientist.

Most of all, I need to thank my advisor Don DeFranco. Don has been a wonderful mentor and teacher over the course of my graduate studies. He has provided measured guidance when it was needed while giving me the independence to pursue my own ideas as other times. Most importantly, he has consistently been a tireless advocate of my cause. I could not have asked for a better thesis advisor or graduate school experience.

Last, but certainly not least, the undying love and support of my family, amma, thaatha, and ayya, throughout this endeavor and in years past has been essential. I am fortunate to have been raised in such a nurturing and intellectually stimulating environment. None of this would have been possible without your support and encouragement. I love you all very much, thank you.

A number of abbreviations are used in this thesis. The major ones are: Connexin 43 (Cx43), glucocorticoid receptor (GR), glucocorticoid (GC), dexamethasone (DEX), gap junction intercellular communication (GJIC), fluorescence recovery after photobleaching (FRAP), and neural progenitor cell (NPC).

1.0 INTRODUCTION

Glucocorticoid hormones (GCs) mediate a wide array of physiological actions following their binding to the glucocorticoid receptor (GR). The principal effects of GCs are mediated by transcriptional responses (i.e. activation or repression) that follow either the direct binding of a GR-ligand complex to glucocorticoid response elements (GREs) contained within target genes, or the indirect association of the receptor with other DNA elements or DNA-bound transcription factors (Haller et al. 2008). However, GR may also act via nongenomic (or non-classical) mechanisms to mediate rapid cellular responses to GCs in the absence of measurable alterations in gene expression (Qiu et al. 2001; Haller et al. 2008; Pasricha et al. 2010). Given the wide use of GCs in a number of clinical settings, such as for the treatment of complications arising from premature birth (Yeh et al. 2004), and the evidence suggesting adverse developmental consequences from GC exposure (Yeh et al. 2004), gaining a full understanding of mechanisms of GC action is of particular importance.

In order to lay the foundations for the particular studies of GC action that I conducted for this thesis, this introduction has been divided into four major sections. Each section focuses on the primary elements of my thesis work. In the first major section, I explore steroid hormones and their receptors with particular attention to GCs and GR. This was the principal focus of my studies. The second section explores gap junctions and the connexin proteins that constitute gap junctions. A major goal of this work was to link GR activation to the modulation of gap junction

intercellular communication (GJIC). In the final two sections of the introduction I summarize some of the relevant literature on caveolin-1 and ERK-1/2, which I hypothesized to be the major signaling proteins linking the activation of GR to changes in GJIC.

1.1 GLUCOCORTICOID HORMONES: THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The physiological release of GC hormones is under the control of the hypothalamic-pituitary-adrenal (HPA) axis. Under conditions of stress, neurons in the paraventricular nucleus (PVN) of the hypothalamus are activated and release corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). CRH and AVP act synergistically to stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which, in turn, acts on the adrenal glands to release GC hormone (Figure 1). In humans the naturally occurring GC is cortisol, while in rodents, it is corticosterone (Groeneweg et al. 2011).

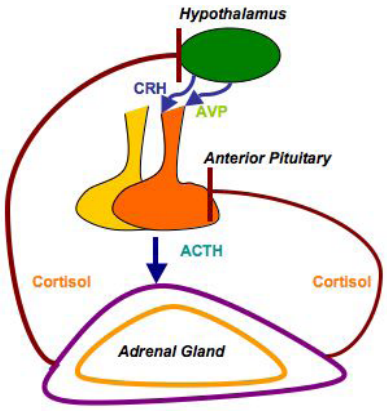

Figure 1. HPA Axis:

CRH and AVP release from the paraventricular nucleus of the hypothalamus stimulates ACTH production in the anterior pituitary gland. ACTH acts on the adrenal gland to stimulate production of cortisol. Cortisol inhibits hypothalamic production of CRH and AVP as well as

ACTH production by the anterior pituitary, thus maintaining homeostasis through a negative feedback loop (Groeneweg et al. 2011).

In addition to the release of GC during periods of physiological and/or psychological stress, there is also a natural circadian rhythm of GC release with hormone levels peaking prior to waking (e.g. 8AM for humans) as well as an underlying ultradian pattern of GC release with a period of approximately 1hr (Young et al. 2004). Disruptions in circadian rhythms have been associated with mood disorders as well as other pathological conditions such as autoimmune diseases (Cutolo et al. 2006; Mendlewicz 2009). Alterations in the circadian rhythms of cortisol secretion have been specifically linked to bipolar disorder as well as rheumatoid arthritis, indicating that the timing of hormone release plays important roles in normal physiology (Cervantes et al. 2001; Cutolo et al. 2006).

GCs bind to both GR and the mineralocorticoid receptor (MR). The MR has a higher affinity for GC than GR. Measurements of GR from CNS tissues revealed a Kd (disassociation constant) for cortisol that was between 2.5-5.0nm while the Kd for MR was approximately 10 fold lower (Reul and de Kloet 1985). Consequently, MR tends to be occupied even when circulating GC levels are at their nadir under physiological conditions (Groeneweg et al. 2011). Furthermore, GR is expressed throughout the brain, whereas MR expression is highest in the hippocampus, is moderately expressed in the amygdala and the prefrontal cortex, and is expressed only at very low levels in other brain regions (Groeneweg et al. 2011).

Another important factor in the selective activation of GR or MR is the expression of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme. The type 2 enzyme inactivates cortisol by converting it to a ketone product (cortisone) (Holmes et al. 2003). The most well characterized role of 11β-HSD2 is in the kidney where it is highly expressed. By inactivating

renal cortisol, this enzyme preserves the ability of MR to be activated only by its primary *in-vivo* ligand, aldosterone. Mutations in the gene for 11β-HSD2 that reduce its activity lead to over-activation of renal MR by cortisol and increased absorption of sodium and water in the nephron, loss of potassium, and hypertension (Holmes et al. 2003). The precise roles of 11β-HSD2 in the developing and adult brain are less well characterized, but the enzyme is known to be expressed in the fetal and, to a much more limited extent, adult, brain, and may impact GC activity. 11β-HSD2 is also highly expressed in the placenta where it acts as a barrier preventing maternal cortisol from having effects on the developing fetus. This enzyme is expressed in the fetal brain starting at around mid-gestation. Expression is turned off post-natally in most brain areas except for the thalamus and cerebellum (Holmes et al. 2003). Importantly, the synthetic GC dexamethasone (DEX), which is commonly used in clinical settings, is a poor substrate for 11β-HSD2 and is therefore capable of crossing the placenta and acting on fetal tissue (Holmes et al. 2003).

Activated GR is capable of producing physiological effects by impacting transcription and by affecting cellular processes independently of transcriptional effects. The following two subsections will focus on these two major mechanisms of GR action.

1.2 GR: CLASSICAL SIGNALING

GR is a ligand-dependent transcription factor belonging to the nuclear hormone superfamily of DNA binding proteins (Cato et al. 2002). As with other members of this superfamily, the amino acid sequence of GR can be divided into 6 major regions. These include a variable N-terminal A/B region, a conserved DNA binding domain (DBD) C region, a linker D region, a conserved

ligand-binding domain (LBD) E region, and a variable F region that lacks a known function (Aranda and Pascual 2001) (Figure 2).

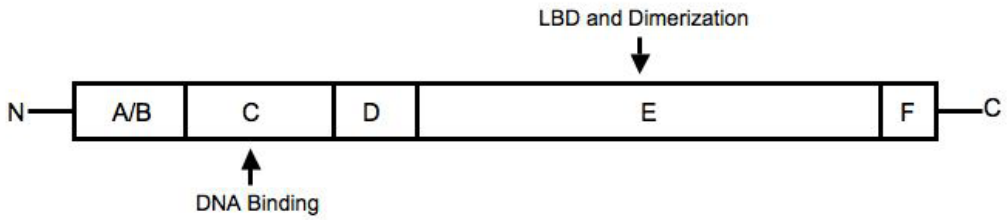

Figure 2: Schematic of a Nuclear Hormone Receptor

Most nuclear hormone receptors, including GR, have 6 domains. The variable A/B domain contains a ligand independent transactivation domain. The conserved C region recognizes specific DNA sequences and is linked to the E domain by a linker D region. The E and F regions contain the ligand binding domain (LBD). In addition, the ligand dependent transactivation domain is proximal to the LBD near the c-terminus of the receptor (Aranda and Pascual 2001). Ligand dimerization of most nuclear hormone receptors occurs through portions of the LBD and the DBD. Ligand-dependent dimerization of GR has been shown to be highly reliant on the LBD and occurs through a distinct dimerization interface in the GR LBD (Bledsoe et al. 2002).

The gene encoding GR contains 9 exons, of which exons 1 and 9 are subject to alternative splicing (Lu and Cidlowski 2006). The two most extensively studied and common isoforms of GR are GRα, which is a ubiquitous, ligand-binding isoform (henceforth referred to simply as "GR"), and GRβ, which does not bind ligand and acts as a negative regulator of GRα activity (Funder 1997; Lu and Cidlowski 2006). GRβ has been shown to act in a dominant negative fashion by competing for co-activators and/or by forming a heterodimer with GRα, thus preventing formation of GRα homodimers that are typically necessary for GRα gene transactivation (Lu and Cidlowski 2006). GR has a high affinity for the synthetic GC dexamethasone (DEX), lower affinity for the naturally produced hormones cortisol and corticosterone and limited affinity for a number of other physiological steroids including aldosterone and deoxycorticosterone (Funder 1997).

According to the "classical," or "genomic," view of nuclear hormone and receptor action, unliganded GR associates with a number of chaperone proteins. These include the heat shock proteins Hsp90 and Hsp70 and the immunophilin FKBP56 (Funder 1997). The chaperone proteins restrict GR to the cytoplasmic compartment (Funder 1997). Hormone binding leads to a disassociation of chaperone proteins, homo-dimerization, and nuclear translocation of the GR-ligand complex (Cato et al. 2002). GR homodimers can then bind to glucocorticoid response elements (GREs) linked to GC responsive promoters in various orientations and positions (Figure 3).

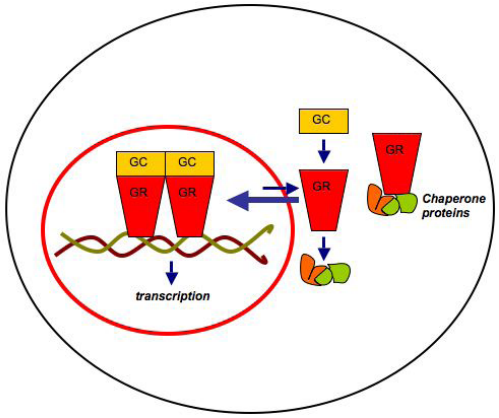

Figure 3: Classical GR Activation

Hormone (GC) leads to disassociation of chaperone proteins from GR, dimerization and translocation of GR to the nucleus. This is a dynamic process, and in the presence of GC, nuclear import of ligand-bound receptor is greater than nuclear export of the receptor. Inside the nucleus, GR dimmers bind to GREs on target DNA leading to transcription of target genes.

The GRE shares a high degree of sequence similarity with the DNA binding sites of other steroid receptors. It consists of two inverted palindromic sequences separated by three nucleotides (Tsai and O'Malley 1994). The prototypical sequence is AGAACA, although there can be a high degree of natural variation from this idealized consensus sequence (Aranda and

Pascual 2001). In fact, progesterone, mineralocorticoid, and androgen receptors (PR, MR, AR) are all capable of binding to the GRE (Tsai and O'Malley 1994). Thus, the specificity of biological responses to the various steroid hormones relies on additional molecular components that are superimposed upon the core recognition by the steroid receptors to their select response elements. For example, the nature of select transcription factors bound in the vicinity of steroid hormone receptors can impact *in-vivo* occupancy of the receptors as well as specific chromatin structural features that influence the specificity of receptor binding in cells.

In addition to activating transcription, the direct binding of GR to distinct sequences termed negative GREs can trigger repression of transcription (Lu and Cidlowski 2006). Alternatively, GR protein can directly interact with other transcription factors such as activating protein 1 (AP-1) and nuclear factor kB (NF-kB) and repress their transcriptional activity (Cato et al. 2002). Although not as well studied, the "tethering" of GR to other DNA-bound transcription factors can also be associated with transcriptional activation.

GR also associates with various non-DNA binding co-regulator proteins that participate in transcriptional regulation. The p160 family of co-activators act as adaptor proteins for binding of additional co-activator proteins (Lu and Cidlowski 2006). Co-activator proteins can also help bind the nuclear receptor to DNA and in many instances also posses a number of enzymatic abilities that aid in regulating transcription. For example, the steroid receptor co-activator (SRC) family has histone acetyltransferase activity that acetylates histones and other proteins at GR promoter sites. CARM1 and PRMT1 are examples of co-activator proteins that are histone methyltransferases (Lonard and O'Malley 2005). Other co-activators are thought to provide some scaffolding function to regulate the assembly of multi-subunit co-regulator complexes. However, recent studies have also found that co-activator proteins have a number of functions apart from

regulating the efficiency of transcription initiation. These functions include mRNA transport and translation, post-translational modifications of the translated proteins, and even cessation of translation by activation of the ubiquitin proteasome pathway (Lonard and O'Malley 2005; Lonard and O'Malley 2006).

1.2.1 GR: Non-Classical Signaling

Steroid hormones have also been shown to have transcription-independent mechanisms of action (Cato et al. 2002; Moriarty et al. 2006). The first report of rapid actions of GCs was by Hans Selye who postulated that GCs may regulate "rapid adaptations to stress" (Selye 1950). In the 1960's, evidence of rapid increases in cAMP following a 15 second pulse of 17-β-estradiol (E2) as well as evidence of E2 binding sites on the surface of endometrial cells provided some of the first evidence of rapid signaling by steroid hormones (Hammes and Levin 2007). This rapid action of steroid hormones is often referred to as "non-genomic" signaling since it is in contrast to direct hormone-receptor activation or repression of transcription (i.e. "genomic" action). However, rapid signaling of this form may eventually lead to genomic effects, for example by activation of signaling cascades that impact transcription and translation. Therefore, the terms "membrane initiated steroid signaling" (MISS) or "non-classical" signaling have more recently been used as more accurate descriptors of this form of hormone action, and I will use the term "non-classical" henceforth (Hammes and Levin 2007).

Non-classical steroid actions are characterized by rapid signaling (typically within a few seconds to 60 minutes), insensitivity to transcriptional and/or translational inhibitors, and continued hormone action despite the use of cell impermeable hormone conjugates (Cato et al. 2002). Non-classical hormone effects may be mediated by the cognate DNA-binding steroid

receptor or through novel hormone-binding receptors that are not members of the nuclear receptor superfamily and possess no inherent DNA-binding activity. In most cases, non-classical effects are characterized by rapid activation of select signal transduction pathways, most commonly mitogen activated protein kinases (MAPKs), adenylyl cyclase (AC), and phosphatidylinositol 3-kinase (PI3K) (Cato et al. 2002).

The most extensively characterized non-classical mechanisms involve estrogen and interaction of this steroid hormone with the major estrogen receptors (ERα and ERβ). Our understanding of ER signaling can serve as a useful model for signaling by other steroid hormones (Cato et al. 2002). Although an area of ongoing debate, most data suggest that non-classical ER signaling is mediated by the classical (i.e., nuclear) ERα and ERβ receptors localized within the plasma membrane (Pedram et al. 2006; Hammes and Levin 2007). In particular, rapid ER signaling is absent in ERα/ERβ knockout mice, and siRNA directed against ERα/ERβ also abrogates rapid ER signaling (Hammes and Levin 2007). Techniques including immunoprecipitation and sucrose gradient fractionation have been used to establish the presence of ER within the plasma membrane and in association with caveolae-containing lipid rafts (Hammes and Levin 2007). It appears that caveolae rafts (discussed further below) provide a physical space where a number of signaling proteins including the steroid receptor, MAPKs, G-proteins, and other molecules can interact (Hammes and Levin 2007).

The membrane localization of ER has been shown to be dependent on palmitoylation of cysteine 447. Mutation of this site prevents plasma membrane localization (Acconcia et al. 2005). Interestingly, this palmitoylation site and the surrounding nine-amino acid domain, which has also been found to be important in promoting palmitoylation, is present (with minor

differences) in GR as well, suggesting that GR membrane localization may be dependent on a similar post-translational lipidation (Groeneweg et al. 2011) (Figure 4).

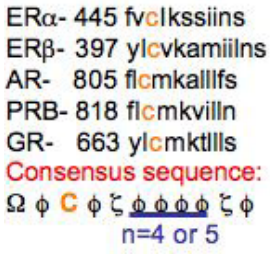

Figure 4: Palmitoylation Sequences in E-Domain of Nuclear Receptors

The consensus palmitoylation domain for ER, AR, PR, and GR are remarkably similar sequences with a cysteine "C" at the three position surrounded by 9-11 amino acids. Key: Ω=aromatic; ϕ=hydrophobic; ζ=hydrophilic. Number represents amino acid number from the beginning of the E-domain (Pedram et al. 2007).

Similar principles of non-classical action have also been demonstrated in GC/GR signaling, although the precise molecular mechanisms remain less well defined (Qiu et al. 2001; Cato et al. 2002). In addition, while a significant body of the ER literature has focused on the role of non-classical ER signaling on cell proliferation and survival, much of the non-classical GR work has focused on rapid effects of GR in modulating the stress response emanating from the central nervous system (CNS) (Groeneweg et al. 2011). There is an ultradian pattern of GC release by the adrenal glands following activation of the HPA axis (Groeneweg et al. 2011). The period of this pattern of troughs and peaks is approximately 1hr and lends itself to rapid effects of GR on target tissue as the circulating levels of GCs drop below the concentration needed to maintain GR activity during troughs (Stavreva et al. 2009; Groeneweg et al. 2011).

Although the focus of GR effects in these studies differs from the focus of this thesis, there appear to be general patterns and principles of rapid, non-classical GR effects that can be instructive. One general principle from these studies is that rapid non-classical GC effects often

seem to be a precursor for more slow acting, but longer lasting, genomic GC effects. In the basolateral amygdala, corticosterone increased the frequency of miniature excitatory postsynaptic currents (mEPSCs) within 15min (Karst et al. 2010). While this rapid effect was found to be non-classical, prolonged GC exposure also increased the frequency of mEPSCs in a manner that was sensitive to protein synthesis inhibitors, and was therefore a genomic/classical effect (Karst et al. 2010). Similarly, in the anterior pituitary, GR activation lead to a decrease in ACTH release within 1 min that was sustained two hours after the GC administration (Groeneweg et al. 2011). The former effect was shown to be non-classical and dependent on the rapid phosphorylation and membrane translocation of annexin-1, whereas the latter was found to be a genomic effect dependent on protein synthesis (Solito et al. 2003; Groeneweg et al.). A second general principle is that the presence of GR (or MR) on the cell surface provides a strong indication for a role for non-classical hormone signaling. A final general principle of rapid non-classical GR signaling is that GC stimulation often acts in a "permissive" manner (Groeneweg et al. 2011). That is, the rapid effects of GCs tend to alter pre-existing activity or the threshold of activity of neurotransmitters and/or ion channels rather than inducing or inhibiting function in an all or none fashion. These insights, as well as knowledge of non-classical signaling from other hormone receptors (such as ER) provide a strong foundation from which to explore whether non-classical GR signaling operates in other neurological systems, particularly during neurodevelopment where rapid GC action remains highly unexplored.

1.3 GR: ROLES IN NEURODEVELOPMENT

GR is known to play a role during neurodevelopment. GR expression begins at around embryonic day 10 (E10) in mice and GC synthesis is detectable by E14 (Reichardt and Schutz 1996). GR mutant mice in which receptor function is deficient have been used to demonstrate that decreased function of the receptor leads to increased mRNA expression of the ACTH precursor proopiomelanocortin (POMC) in the anterior pituitary and increased mRNA expression of CRH in the hypothalamus by E16.5 (Reichardt and Schutz 1996). This suggests that a functional GR is necessary prenatally for the establishment of proper negative feedback loops in the HPA axis. This finding has been strengthened by more recent analyses using a GR conditional knockout mouse where Cre recombinase was under the control of a CamKIIα promoter. This led to a loss of GR in neurons and glial cells in the developing brain (including in the pituitary gland) and over a 750-fold increase in the expression of plasma corticosterone and a 16-fold increase in plasma ACTH. These mice did not survive past postnatal day 10 (Erdmann et al. 2008). The effects of prenatal HPA axis disorders are thought to have effects on physiology and behavior that extend beyond the prenatal period. For example, dysregulation of the HPA axis prenatally is hypothesized to contribute to mood disorders and other psychiatric conditions such as schizophrenia later in life (Reichardt and Schutz 1996).

1.3.1 Prenatal Stress

The importance of regulating GR activity during fetal development is also demonstrated by the high level of 11β-HSD2 expression in the placenta as well as in the fetal brain beginning at mid-gestation (Holmes et al. 2003). One implication of this finding is that high levels of GC,

including in the developing brain, may have deleterious effects on fetal development. This may be prevented by 11β-HSD2 expression. Therefore, many studies have focused on the effect of "prenatal stress" or exogenous antenatal GC administration on neurodevelopment (Glover et al. 2009). Specifically, these studies aimed to understand the effect of supraphysiologic GC levels (levels sufficient to overwhelm or bypass 11β-HSD2) on fetal brain development. Although there is some variability in outcomes, both human and animal studies of prenatal stress suggest that exposure of fetuses to abnormally elevated levels of GCs leads to an increase in the stress response postnatally and generally deleterious effects on neurodevelopment (Glover et al. 2009). For example, exposing female non-human primates to unpredictable noise during pregnancy led to a reduced volume of the hippocampus in offspring (Coe et al. 2003). Rodents exposed to prenatal stress produced offspring with lower levels of both GC receptors (i.e. MR and GR) (Glover et al. 2009). The decrease in MR and GR may partly explain the heightened stress response in offspring of mothers exposed to prenatal stress since feedback inhibition is a critical source of regulation of the HPA axis.

While prenatal stress can have a number of physiological effects on the pregnant mother and her offspring, the importance of GC activity in producing some of the observed effects in the CNS has been strengthened by experiments using adrenalectomized dams. Since the adrenal glands produce GC hormones, adrenalectomy prevents GC release. Barbazanges *et al*. showed that the pups of adrenalectomized dams subject to prenatal stress did not show the decrease in hippocampal GR levels seen in pups from dams with intact adrenals or in pups from adrenalectomized dams given corticosterone injections (Barbazanges et al. 1996).

The results of human clinical studies of pregnant mothers exposed to prenatal stressors are varied, but prenatal stress has been commonly associated with an increased incidence in

childhood attention deficit hyperactivity disorder (ADHD)(Glover et al. 2009). A general increase in anxiety, decreased cognitive development, and delayed language development are among the other outcomes in children of mothers exposed to prenatal stressors (Glover et al. 2009).

1.3.2 Exogenous Antenatal GC Administration

Prenatal stress is also likely to involve other hormones in addition to GCs, so caution must be applied in ascribing neurodevelopmental defects to altered GR signaling alone. A more direct method of identifying GR effects is through an examination of the clinical effects of exogenous GC administration. Neurodevelopmental defects have also been observed when synthetic GCs, such as DEX or betamethasone, are administered exogenously to pregnant females in the absence of underlying prenatal stress. This is a clinically relevant application since DEX and betamethasone are administered to pregnant mothers for the treatment of complications of prematurity and for congenital adrenal hyperplasia (CAH) (Yeh et al. 2004; Vos and Bruinse 2010). Antenatal exposure of pregnant rats to DEX led to decreased learning and memory in offspring and an increased sensitivity of the pups' hippocampal and cortical neurons to injury following a toxic insult (Emgard et al. 2007). Similarly, administration of DEX to pregnant rats led to impaired radial migration of neural progenitor cells in the embryos (Fukumoto et al. 2009). Taken in combination with the prenatal stress studies, this work strongly suggests that exposure of embryos to elevated GCs impairs CNS development and function and has negative effects postnatally on cognition and behavior. The precise molecular mechanisms underlying these GC effects remain less clear, although there have been a number of important insights from recent *in-vitro* and *in-vivo* studies that will be described below.

1.3.3 GR: Cellular Effects

At the cellular level, GCs have been shown to affect both the proliferation and differentiation of cells in the developing CNS (Sabolek et al. 2006; Sundberg et al. 2006). Alteration of the pattern of cell differentiation commonly results from GC treatment. For example, exposure of rat neural progenitor cells (NPCs) to a high dose of DEX (10uM) was shown to decrease astroglial differentiation (Sabolek et al. 2006). Similarly, DEX exposure of a neuroblastoma cell line resulted in increased differentiation into neuroendocrine cells (Ross et al. 2002). *In-vivo* and *in-vitro* studies by Sundberg and colleagues demonstrated that exposure to GCs decreased NPC proliferation by reducing the levels of the cell cycle protein cyclin D1 (Sundberg et al. 2006).

The detrimental effects of GC exposure on NPC proliferation have also been demonstrated in the context of adult neurogenesis. The subgranular zone is one of the major sites of adult neurogenesis. Chronic stress and/or GC administration has been shown to significantly decrease neurogenesis in the subgranular zone of the adult rat hippocampus (Schoenfeld and Gould 2011). In contrast, adrenalectomy in adult rats resulted in increased neurogenesis of subgranular neurons (Schoenfeld and Gould 2011). GC-mediated decreases in NPC proliferation and alteration of differentiation may account for some of the observed behavioral and neuro-developmental consequences of GC exposure. The above studies provide some insights into mechanisms of GC action in the developing brain, but many aspects of NPC response to these hormones remain to be explored.

1.4 GC: CLINICAL USE AND CLINICAL EFFECTS

Synthetic GCs such as DEX are used in various clinical contexts. DEX administration is commonly used throughout pregnancy beginning at 5-6 weeks of gestation to mothers of children at high risk for congenital adrenal hyperplasia (CAH) (Vos and Bruinse 2010). This is a condition marked by inadequate cortisol and aldosterone production, excess testosterone production, and resultant virilization of female fetuses (Hirvikoski et al. 2007). In most cases, CAH is caused by a deficiency in the 21-hydroxylase enzyme, which is necessary for the production of both cortisol and aldosterone. Cortisol is necessary for feedback inhibition at the pituitary gland. Since pituitary signals also determine testosterone production, the lack of cortisol-mediated feedback inhibition leads to excess testosterone levels and virilization of female fetuses (Vos and Bruinse 2010). DEX administration early in pregnancy inhibits pituitary-mediated production of testosterone and prevents virilization (Hirvikoski et al. 2007).

DEX is also administered antenatally to accelerate heart and lung development in pregnancies at high risk for preterm delivery, and postnatally in the treatment and prevention of respiratory distress syndrome (Karlsson et al. 2000). A widely cited study by Yeh *et al.* evaluated cognitive development in infants that were given DEX for the treatment of severe respiratory distress syndrome. This was a double-blind placebo controlled study in which DEX was administered in a tapered dose for 28 days following premature birth. The infants were given their first dose of DEX within 12hrs of birth and their neurological function and IQ was assessed between ages 7-9 years by a pediatric neurologist or a teacher (for IQ scores) who was blinded to the study design or the clinical history of the children. Of the 262 infants that received treatment, 146 were evaluated at school age. The clinical assessment indicated a variety

of negative neurobiological side effects of DEX including significantly decreased motor skills, significantly impaired motor coordination, and significantly decreased IQ (Yeh et al. 2004).

In a separate study, decreased cortical folding was found on MRI in infants that received antenatal GCs (Modi et al. 2001). Since CAH is a relatively rare condition, fewer data are available on the effects of DEX treatment on the neuro-development of these children. Based on the available data, however, a number of clinical reviews express concern about the potential negative neurodevelopmental consequences of early prenatal DEX treatment, and at least one recent clinical assessment found decreases in verbal working memory, self-perception of scholastic competence, and increased self-rated social anxiety in 7-17 year olds that received antenatal DEX for CAH (Miller 1999; Modi et al. 2001; Hirvikoski et al. 2007).

In adults, exposure to chronic levels of endogenous stress hormones such as the GC cortisol is associated with mood disorders and cognitive deficits that may be linked to hormone effects on cell proliferation (McEwen 2008). Specifically, these illnesses have been partly attributed to GC inhibition of adult neural stem/progenitor (NSC/NPC) cell proliferation (Mirescu and Gould 2006). One of two major sites of adult neurogenesis is in the dentate gyrus (DG) of the hippocampus. Chronic stress has been shown to lead to hippocampal atrophy that is also correlated with increased incidence of depression as well as deficits in learning and memory (McEwen 2004). Stress-induced suppression of cell proliferation in the DG of the hippocampus has been observed in a number of different mammals, including rats and mice, in response to a variety of stressors including footshock, restraint stress, and predator odor (Mirescu and Gould 2006).

GCs have specifically been cited as important in the reduction of cell proliferation from these stress-inducing manipulations because adrenalectomy or blockade of HPA axis receptors

(such as CRF-1) increase cell proliferation (Mirescu and Gould 2006; Schoenfeld and Gould 2011). The inhibitory effect of GCs on DG cell proliferation is likely mediated through GR and not MR. Even though both of these GC receptors exist in the adult hippocampus, the high affinity MR is occupied under baseline (unstressed) levels of GC circulation, unlike GR (Mirescu and Gould 2006). Furthermore, pharmacological blockade of GR in rats prevented the loss of cell proliferation produced by elevated corticosterone levels (Wong and Herbert 2005). Based on these clinical findings, gaining a better understanding of the mechanisms that underlie GC effects on NPC proliferation has the potential to have far-reaching clinical implications for a number of diseases that affect both adults and children.

One potential GC target that may impact NPC proliferation are intercellular channels termed gap junctions and the connexin proteins that constitute these channels. Connexins have been previously shown to have effects on NPC proliferation and connexin expression and function may be altered by GR activity (Cheng et al. 2004).

1.5 GAP JUNCTIONS AND THE GAP JUNCTION PROTEINS: GENERAL BIOLOGY

Gap junctions form intercellular channels between adjacent cells that allow the passage of ions and molecules less than 1kD in size (Bruzzone and Dermietzel 2006). Mammalian gap junctions are composed of connexin proteins, six of which make up a single connexon, or hemichannel (Bruzzone and Dermietzel 2006). Two opposing hemichannels on adjacent cells, in turn, constitute a gap junction (Goodenough and Paul 2003) (Figure 5).

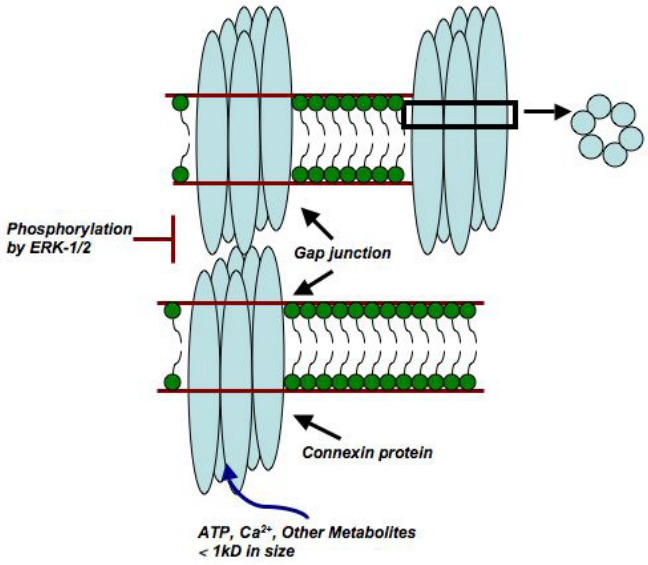

Figure 5: Gap Junctions are Composed of Connexin Proteins

Six individual connexin proteins combine to form a gap junction hemi-channel. Two hemi-channels from adjacent cells form a gap junction through which metabolites and small molecules less than 1kD in size can pass. Connexin 43 gap junctions can be phosphorylated by ERK-1/2, and this phosphorylation leads to inhibition of gap junction intercellular communication (Laird 2006).

Over 20 connexin genes have been identified and classified according to predicted molecular weight. Furthermore, expression of the various connexins is highly tissue specific (Bruzzone and Dermietzel 2006). The most ubiquitous and most extensively studied connexin is connexin 43 (Cx43) (Laird 2006).

Connexins are integral membrane proteins, which pass through the cell membrane four times. The connexin proteins have two extracellular loops and one intracellular loop with their N and C-termini exposed to the cytoplasm (Laird 2006). These proteins have a relatively short lifespan, measured to be as short as 1hr, thus providing one potential mechanism whereby levels of gap junction intercellular communication (GJIC) can be rapidly modified (Laird 2006). Most connexins are co-translationally inserted into the endoplasmic reticulum (ER) and have been reported to oligomerize in the ER as well (Laird 2006). Following ER exit, connexins typically

pass through the *cis*-Golgi network and are then inserted into the plasma membrane where they diffuse inside lipid bilayers as connexons. The formation of gap junctions appears to require association of connexons with N- and E- cadherins that allow for the docking of connexons/hemi-channels on adjacent cells into a functional gap junction (Laird 2006).

Gap junction degradation generally begins with the internalization of double membrane vesicular structures called "annular junctions." These structures are an internalized gap junction or part of a gap junction. In some cases, such as has been demonstrated for Cx43, gap junctions may be internalized in smaller segments via clathrin coated pits or via a caveolin-dependent process. Internalized plaques are usually subject to degradation in lysosomes. However, while most connexin proteins leaving the plasma membrane are mono-ubiquitinated and subject to lysosomal degradation, there is also evidence suggesting that internalized connexin proteins are subjected to poly-ubiquitination and proteasomal degradation (Laird 2006).

1.5.1 Gap Junctions: Post-Translational Modification by Phosphorylation

Most connexins are subjected to post-translational modification by phosphorylation. For example, the C-terminal domain of Cx43 has a number of sites that can be phosphorylated. In general, phosphorylation of Cx43 leads to inhibition of GJIC (Saez et al. 2003; Ai and Pogwizd 2005). Several kinases including Protein Kinase A (PKA), Protein Kinase C (PKC), Src, and MAPK have been shown to phosphorylate Cx43 (Solan and Lampe 2009). Phosphorylation of Cx43 leads to a change in the electrophoretic migration of the protein through SDS/PAGE gels. In the absence of phosphorylation, or when cell lysates are subject to alkaline phosphatase treatment, only a single "P0" band is detected. The slower migrating "P1" and "P2" bands are likely to be phospho-isoforms of Cx43. The electrophoretic isoforms are thought to be a product

of both the additional mass of phosphorylation (80Da) as well as conformational changes in the protein that resist denaturation even under SDS-PAGE conditions (Solan and Lampe 2009).

The effect of phosphorylation on Cx43 is highly dependent on the specific kinase responsible for the phosphorylation and, in turn, the specific site of phosphorylation (Solan and Lampe 2009). For example, PKA enhances Cx43 delivery to the plasma membrane and gap junction plaque assembly, whereas PKC phosphorylation at serine 262 inhibits cell cycle progression of proliferating cardiomyocytes (Paulson et al. 2000; Doble et al. 2004). ERK-1/2, a member of the MAPK family, is an important regulator of Cx43 phosphorylation and has specifically been shown to inhibit GJIC following phosphorylation of serines 279, 282, and 255 on the Cx43 protein (Warn-Cramer et al. 1998). Other major regulators of Cx43 phosphorylation include the protein serine/threonine phosphatases (PSTPs) such as protein phosphatases 1 (PP1) and 2A (PP2A) (Cruciani et al. 1999; Saez et al. 2003; Ai and Pogwizd 2005). Cx26, unlike most other gap junction proteins, is not known to be modified by phosphorylation. Regulation of its function may therefore be mediated at the level of gene expression. In fact, one report demonstrated that Cx26 is transcriptionally upregulated by GCs (Kojima et al. 1995).

1.5.2 Gap Junctions: Expression in the CNS

Of the 20 connexin subtypes identified, at least five connexins are known to be expressed in the rodent cerebral cortex (Bruzzone and Dermietzel 2006; Elias and Kriegstein 2008). While there are clear temporal and spatial variations in the expression of connexin isoforms within the CNS, both Cx43 and Cx26 are localized to proliferating and undifferentiated neurons (Nadarajah et al. 1997; Bruzzone and Dermietzel 2006). Specifically, Cx26 is expressed throughout the developing rodent brain from the ventricular zone, through the intermediate zone and up to the

cortical plate, whereas Cx43 is most highly expressed in the ventricular zone and is highly expressed *in-vitro* in NPC cultures (Nadarajah et al. 1997; Cheng et al. 2004; Elias et al. 2007).

In the adult brain, Cx36 is the most abundantly expressed connexin in neurons. Cx26, Cx43, and Cx30 are all expressed in astrocytes, and Cxs 29, 32, and 47 are expressed in oligodendocytes (Connors and Long 2004). Cx36 has been found to play an important role in electrical gap junction coupling in adult CNS neurons. The physiological importance of gap junction mediated coupling in neurons was primarily thought to involve the maintenance of subthreshold spiking among groups of neurons (Connors and Long 2004). More recent findings have suggested that gap junction electrical synapses have specific effects on behavior, such as contributing to fear learning and memory in rats (Bissiere et al. 2011). In contrast to neurons, the precise role of gap junctions in normal physiology in astrocytes and oligodendrocytes is controversial, but the passage of Ca^{2+} currents, buffering of K^{+}, and the intercellular passage of other small metabolites such as ATP remain the favored theories (Orthmann-Murphy et al. 2008).

1.5.3 Gap Junctions and NPC Proliferation

Gap junctions and their constituent connexin proteins may play a number of important roles in the development of the embryonic brain (Bruzzone and Dermietzel 2006; Elias and Kriegstein 2008). Gap junction coupling has been demonstrated during most stages of embryonic cortical development and remains prominent during the early postnatal period as well (Bittman et al. 2002). Pharmacologic disruption of GJIC in neural progenitors has been shown to decrease the rate of proliferation and prevent cells from entering the cell cycle (Bittman et al. 2002). Inhibition of Cx43 gap junctions in *in-vitro* NPC culture led to decreased proliferation and

increased differentiation of these cells. In addition, NPCs remained in a proliferative state following basic fibroblast growth factor (bFGF) withdrawal if Cx43 was simultaneously overexpressed, indicating that Cx43 mediated GJIC may be necessary for NPC proliferation (Cheng et al. 2004).

The specific mechanism whereby gap junctions facilitate NPC proliferation remains an active area of investigation. One area of focus has been the role of gap junctions and hemichannels in the propagation of Ca^{2+}. Intercellular Ca^{2+} signaling was significantly decreased following pharmacological inhibition of GJIC in coupled HEK293 cells (Toyofuku et al. 1998). In addition, the propagation of spontaneous Ca^{2+}waves through a Cx43 hemichannel-dependent process was shown to be necessary for the proliferation of radial glial cells in the embryonic ventricular zone (Weissman et al. 2004). In addition, gap junction-mediated passage of small molecules such as cAMP or cell cycle proteins have also been posited to influence cell proliferation (Huang et al. 1999; Tabernero et al. 2006). More recent work has also suggested that Cx26 and Cx43 may act as adhesive proteins facilitating radial glial migration during embryonic cortical development (Elias et al. 2007). According to these studies, decreased cell numbers that are observed following gap junction inhibition or knockout during cortical development may be partly explained by connexin-dependent deficiencies in progenitor cell migration. Observations from human diseases resulting from mutations in connexins have also been instructive in illustrating the potential role of these proteins in proliferation.

1.5.4 Gap Junctions in Disease: Oculodentodigital Dysplasia

Mutations in various connexins are associated with at least eight human diseases (Laird 2006). The only human disease associated with a Cx43 mutation, which is the most ubiquitous and

extensively studied connexin, is ococulodentodigital dysplasia (ODDD). At least 28 different mutations in the Cx43 gene have been associated with ODDD (Laird 2006). Patients suffering from this autosomal dominant mutation have a combination of brittle nails, hair abnormalities, conductive hearing loss, lens defects, corneal defects, abnormalities of the teeth, and variable neurological and cardiovascular abnormalities (Loddenkemper et al. 2002; Laird 2006). The neurological symptoms of ODDD include motor disorders (in particular spasticity) autonomic dysregulation, bladder control issues, cranial nerve abnormalities, and some cases of mental retardation or reduced intelligence (Loddenkemper et al. 2002). In addition, subcortical white matter lesions and basal ganglia changes have been reported on MRI from ODDD patients (Loddenkemper et al. 2002).

In-vitro analysis of two mutant Cx43 variants associated with ODDD revealed that mutant Cx43-expressing cells form gap junctions that lack GJIC as measured by Lucifer yellow dye coupling (Roscoe et al. 2005). In addition, co-expression of WT and mutant Cx43 revealed that the mutant protein acts in a dominant negative fashion and down-regulates GJIC (Roscoe et al. 2005). The loss of GJIC has also been shown in a mouse model of ODDD where GJIC in granulosa cells from these mice showed an 80-90% reduction in GJIC compared to WT cells (Flenniken et al. 2005).

Aspects of these neurological findings are further corroborated by a Cx43 conditional knockout (cKO) mouse. This cKO mouse, termed “Shuffler” due to characteristic abnormal gait and ataxia reminiscent of the ODDD phentoype, has Cre recombinase driven by a GFAP promoter leading to loss of Cx43 in premitotic radial glial cells and mature astrocytes (Wiencken-Barger et al. 2007). The shuffler mouse was found to have disorganization and decreased size of the cortex, cerebellum, and hippocampus, as well disorganization of the

ventricular and subventricular zones (Wiencken-Barger et al. 2007). The findings from the Shuffler mouse suggest that defects in neural progenitor proliferation and/or migration may account for some of the phenotypic abnormalities seen in the adult animal.

Taken in combination with the literature on GR, there is therefore a substantial body of evidence suggesting that connexin proteins and GR can modulate cell proliferation. The proteins and signaling cascades that link GR and connexins to cell proliferation are, however, less well defined. One protein that may play a critical role in the facilitating GR and connexin crosstalk is caveolin-1.

1.6 CAVEOLAE AND CAVEOLIN-1

Caveolae are specialized membrane invaginations localized to sphingolipid-rich domains called lipid rafts (Langlois et al. 2008). Caveolae have a role in diverse cellular functions including endocytosis, transcytosis, calcium signaling, and the facilitation of various signal transduction pathways (Parton and Simons 2007). The major proteins that constitute caveolae are caveolins. There are three isoforms of caveolin (cav) in mammalian cells (cav-1, 2, and 3) (Quest et al. 2004). Caveolins are membrane proteins with N and C termini in the cytoplasm and a hairpin intermembrane domain (Parton and Simons 2007). Cav-1/cav-2 caveolae form following oligomerization of the caveolins and association with cholesterol in lipid rafts (Parton and Simons 2007).

Cav-1 and cav-2 are typically co-expressed in various tissues, whereas cav-3 is expressed in a homomeric form and is usually limited to muscle cells (Quest et al. 2004). Despite co-expression of cav-1 & 2 in most contexts, a cav-2 knockout (KO) in mice has few physiological

effects and does not alter the morphology or number of caveolae (Quest et al. 2004). In contrast, a cav-1 KO leads to a complete loss of caveolae formation in tissues expressing cav-1/cav-2 and to more profound pathophysiological changes in many tissues (Quest et al. 2004; Jasmin et al. 2009). For example, cav-1 KO mice exhibit cardiac hypertrophy most likely through activation of MAPK signaling, defects in angiogenesis including reduced blood vessel density and incompletely formed capillaries, microvascular hyperpermeability due to defects in tight junctions and in the attachment of endothelial cells to the basement membrane, and reduced lifespan (Hnasko and Lisanti 2003).

1.6.1 Caveolins: Roles in Signal Transduction

Caveolins are thought to play a central role in signal transduction pathways originating from the cell surface (Parton and Simons 2007). For example, cav-1 has been proposed to act in an anti-proliferative manner in CHO cells by down-regulating ERK-1/2 signaling (Quest et al. 2004). Along the same lines, knockdown of cav-1 was shown to increase ERK-1/2 activity and induce tumorogenecity in NIH-3T3 cells (Galbiati et al. 1998). Caveolin-1 has also been shown to limit cellular proliferation by other mechanisms such as sequestering β-catenin to the plasma membrane thereby preventing the transcription of pro-proliferative genes such as cyclin D1 (Quest et al. 2004). While in most contexts cav-1 expression is associated with decreased proliferation, cav-1 was shown to enhance cell survival and proliferation of prostate cancer cells (Li et al. 2009).

The precise mechanisms whereby cav-1 expression can be pro-proliferative in one context but anti-proliferative in another remain unclear. However, cav-1 may play a role in intercellular signaling through effects on GJIC (Langlois et al. 2008; Matthews et al. 2008).

Specifically, cav-1 has been found to co-immunoprecipitate with Cx43, and overexpression of cav-1 has been shown to increase GJIC (Matthews et al. 2008).

1.6.2 Caveolins: Cav-1 and Steroid Hormone Receptors

Cav-1 has also been implicated in signal transduction pathways involving steroid hormone receptors. The most well established case involves the facilitation of membrane ER signaling. In this case, immunoprecipitation studies established an association between cav-1 and ERα. Furthermore, biochemical fractionation and indirect immunofluorescence studies revealed the presence of ERα within the plasma membrane (Razandi et al. 2002). In addition, over-expression of cav-1, and in particular the cav-1 scaffolding domain, increased ERα membrane localization, suggesting that cav-1 expression directs membrane localization of the ER (Razandi et al. 2002).

Serine 522 in the E-domain of ERα was found to be particularly important for association with cav-1. Mutation of this serine to alanine reduced cav-1 association with ERα by 60%, but had no effects on nuclear localization or transcriptional activity of the receptor. In contrast, mutations in the A/B or C domains of ERα had no effects on association of the receptor with cav-1 (Razandi et al. 2003). Interestingly, E2 stimulation decreased ERα association with cav-1 in MCF-7 breast cancer cells, but increased this association in vascular smooth muscle cells, providing a potential mechanism to explain how cav-1 expression may have differential effects in different cell types (Razandi et al. 2002).

More recently, exploration of membrane localized GR has revealed similar patterns of association with cav-1. In particular, GR and c-src were found to co-localize to cav-1 enriched

membrane fractions in A549 lung epithelial cells and knockdown of cav-1 was shown to limit a GC and c-src dependent non-classical GR signaling mechanism (Matthews et al. 2008). In particular, cav-1 knockdown prevented GR dependent activation of protein kinase B and reduced GR-mediated inhibition of cell proliferation (Matthews et al. 2008). Taken together with cav-1's potential involvement in GJIC (see above), these findings suggest a potential, and heretofore unexplored, molecular mechanism whereby non-classical GR actions may be coupled to downregulation of GJIC via a cav-1 dependent process.

According to this hypothesis, cav-1 links GR action to connexin through its role as a structural protein that maintains caveolae-containing lipid rafts. However, this does not explain how GR activation alters connexin proteins. One signaling cascade that may link GR and connexin in a cav-1 dependent manner is the ERK-1/2 pathway.

1.7 ERK-1/2 SIGNALING: BASIC BIOLOGY

ERK-1/2 belongs to the MAPK family and is a key part of a cellular signaling cascade that transmits signals from the cell surface to the nucleus (Seger and Krebs 1995; Galabova-Kovacs et al. 2006). The general scheme of signal transduction leading to ERK-1/2 activation (i.e., phosphorylation) begins when cell surface signaling, typically from growth factor receptors, initiates the small G protein Ras (Seger and Krebs 1995) (Figure 6).

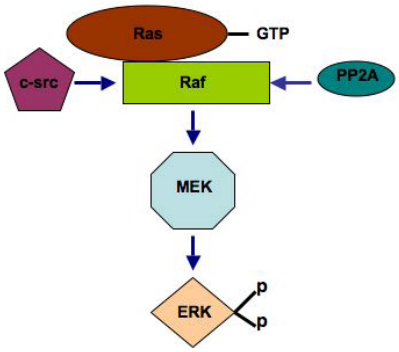

Figure 6: Schematic of an ERK-1/2 Activation Pathway

ERK-1/2 activation may occur in response to a diverse array of upstream signals and can be regulated by the interaction of a number of kinases and phosphatases. Figure 6 illustrates a simplified version of one activation pathway. The small G-protein Ras is activated into Ras-GTP by cell surface signaling. Ras binds to and activates Raf. Phosphorylation of certain Raf isoforms by kinases such as the c-src family as well as dephosphorylation by phosphatases such as PP2A aid in its activation. Activated Raf phosphorylates and activates MEK, which then phosphorylates and activates ERK (McCubrey et al. 2007).

Ras is able to activate a number of signaling pathways including Raf/MEK/ERK, PI3K/Akt, and RalEGF/Ral (McCubrey et al. 2007).

There are four Ras proteins and they each have varying abilities to activate the three downstream signaling cascades outlined above, with the K-Ras subtype of Ras thought to be the stronger activator of the Raf/MEK/ERK pathway (McCubrey et al. 2007). Either farnesylation or geranylgeranylation at a cysteine residue on Ras are necessary for its recruitment to the plasma membrane and subsequent activation. Upon activation by growth factors, mitogens, or cytokines, active Ras recruits Raf from the cytoplasmic compartment to the cell membrane (Galabova-Kovacs et al. 2006).

The Raf proteins, which consist of A-Raf, B-Raf, and C-Raf (or Raf-1) are serine/threonine kinases which contain a number of regulatory phosphorylation sites. Recruitment by Ras, dimerization, and phosphorylation/dephosphorylation on various sites are

all key events in activation of Raf (McCubrey et al. 2007). In particular, dephosphorylation at serine 259 by PP2A and phosphorylation at tyrosines 340 and 341 by Src family kinases are key in Raf activation (Chang et al. 2003). Activated Raf phosphorylates the S/T dual-specificity mitogen activated protein kinase/ERK kinase (MEK) on serine residues in the MEK catalytic domain (McCubrey et al. 2007). MEK, in turn, phosphorylates and activates ERK-1/2 (Galabova-Kovacs et al. 2006).

1.7.1 ERK-1/2 Signaling: Downstream Targets and Effects

Once activated, the Ras/Raf/MEK/ERK pathway has a number of downstream targets and consequently has been shown to have wide ranging effects on cell physiology including modulation of cell proliferation, migration, survival, and differentiation (Galabova-Kovacs et al. 2006). Proliferation is a particularly common outcome from Ras/Raf/MEK/ERK signaling and, in fact, constitutive activity of Ras proteins has been found in around 30% of human cancers (Chang et al. 2003). Interestingly, there are also some instances where Ras activity has been shown to have anti-proliferative activity (McCubrey et al. 2007). One explanation for this difference may be that different Raf proteins activated by Ras activate different targets. For example, transfecting NIH-3T3 cells with A-Raf led to cyclin D1 up-regulation and an increase in proliferation. In contrast, transfection of NIH-3T3 cells with B-Raf led to induction of p21 and G_1 arrest (Chang et al. 2003).

In addition to direct effects of Raf on cell proliferation, activated ERK-1/2 itself phosphorylates and activates targets such as the transcription factors Ets-1, AP-1, c-Myc, NF-kB, and CREB, all of which can have effects on cell proliferation (Chang et al. 2003). A number of these ERK-1/2 targets induce pro-proliferative genes such as cyclins and cyclin dependent

kinases. For example, CREB, Ets-1, and AP-1 all have been shown to directly or indirectly lead to increased expression of the pro-proliferative gene, cyclin-D1 (McCubrey et al. 2007). However, in some cases these transcription factors may also inhibit proliferation by inducing genes such as p21 that can cause cell cycle arrest (Chang et al. 2003). In the specific case of neural progenitor/stem cells, the available evidence also suggests that ERK-1/2 can act in a pro- or anti-proliferative (pro-differentiation/pro-apoptotic) manner. For example, fibroblast growth factor-2 (FGF-2) dependent proliferation of adult NPCs was shown to depend on ERK-1/2 mediated up-regulation of cyclin D1 (Kalluri et al. 2007). Similarly, the insulin like growth factor-1 (IGF-1) mediated proliferation of oligodendrocyte progenitor cells was shown to depend on ERK-1/2 activation (Cui and Almazan 2007).

ERK-1/2 activation has also been shown to be important for the differentiation of NPCs. For example, an increase in ERK-1/2 activity correlated with bone morphogenic protein 4 (BMP4) mediated differentiation of rat NPCs (Moon et al. 2009). In the context of mature neurons, ERK-1/2 activation has been shown to act as a pro-apoptotic factor. ERK-1/2 activation has also been shown to increase neuronal cell death, such as in the context of oxidative stress (Levinthal and Defranco 2005). Taken together, these findings suggest that the Ras/Raf/MEK/ERK pathway, and more specifically ERK-1/2, has a highly context and cell-type dependent role in proliferation.

1.7.2 ERK Signaling: GR and Gap Junctions

A more recently identified target of ERK-1/2 that may also have potential effects on NPC survival and/or proliferation is the Cx43 protein. In particular, serines 279, 282, and 255 (S279/S282) and (S255) in the carboxyl tail of Cx43 have been identified as consensus ERK-1/2

target sites (Warn-Cramer et al. 1998; Cameron et al. 2003). Phosphorylation of Cx43 S279/S282 and S255 was shown to down regulate GJIC (Warn-Cramer et al. 1998). In light of the previously described relationship between changes in GJIC and effects on cell proliferation, examining ERK-1/2 effects on Cx43 phosphorylation may potentially provide unique insights into cell cycle progression in GC-exposed NPCs. Interestingly, GR has been shown to rapidly activate ERK-1/2, providing a potential mechanism linking GC stimulation to modulation of GJIC and cell proliferation (Qiu et al. 2001; Cato et al. 2002).

1.8 DEX TREATED PRIMARY NEURAL PROGENITORS AS A MODEL SYSTEM FOR GC EFFECTS ON GJIC AND CELL PROLIFERATION

In order to experimentally determine the nature of the interaction of GR and GJIC in the context of neurodevelopment, and to precisely characterize the potential role of cav-1 and ERK-1/2 in this process, we needed an appropriate model system. DEX treated primary NPCs were highly suitable for this task.

Embryonic day 14 (E14) derived primary mouse NPCs are an ideal platform for studying the role of connexin proteins in proliferation and their regulation by GCs. The Cx43 protein is expressed in embryonic derived NPCs, and we have demonstrated the presence of both Cx43 and Cx26 in our NPC cultures (i.e., neurospheres) (Cheng et al. 2004). In addition, GR is expressed in the embryonic brain prior to E14 and is also expressed in the primary NPCs used in our laboratory (Reichardt and Schutz 1996). It has been previously shown that DEX administration limits primary NPC proliferation in a GR-dependent fashion (Sundberg et al. 2006). This system therefore expresses all of the critical cellular components we were interested in studying and is

capable of recapitulating the primary physiological effect of hormone exposure (i.e., decreased proliferation) that we want to study. In addition, an *in-vitro* system is highly manipulable and allows us to easily use a combination of imaging techniques such as FRAP, biochemical techniques such as Western blot, and molecular biological techniques such as qRT-PCR.

1.9 DISSERTATION GOALS

The major objectives of the work presented in this thesis are as follows: (1) to understand the effects of a transient GC exposure on neural progenitor cell gap junction intercellular communication, (2) to explain the molecular mechanism whereby a transient hormone exposure could affect GJIC, and (3) to determine the impact of this brief GC exposure on cell physiology. I was motivated to pursue these objectives based on a number of previous studies (referenced in my introduction) that provided the following important insights: First, that GC exposure limits NPC proliferation *in-vitro* and *in-vivo*. Second, that changes in connexins and GJIC can, in turn, modulate the proliferation of NPCs and other cell types *in-vitro*. Third, that transient GC exposure activates signaling cascades, including the MAPK pathway, and that these signaling cascades can impact connexin protein and GJIC. Fourth, and finally, that human clinical studies suggest that antenatal or postnatal exposure to synthetic GCs such as DEX may have adverse consequences on neurodevelopment. Based on these findings, I hypothesized that a transient GC exposure would limit GJIC, and that this reduction in intercellular communication would, in turn, reduce cell proliferation.

To test these hypotheses I first adapted the gap-FRAP method for measuring GJIC in neurospheres. These studies revealed that a 1hr DEX exposure significantly reduced GJIC in

NPCs in a GR-dependent manner, and comprised the fundamental physiological observation that motivated my subsequent studies. The majority of the experiments that followed were aimed at understanding the molecular basis for this loss of GJIC. I focused my studies on the expression and phosphorylation of the connexin proteins that constitute gap junctions and potential signaling cascades that could lead to rapid alterations in connexins, and, as a result, GJIC. Finally, in order to understand the broader consequence of a reduction in GJIC on NPC physiology, I examined the effect of a transient loss in GJIC on subsequent NPC proliferation. These studies have revealed a non-classical GR-mediated mechanism with rapid GC effects on GJIC that is mediated by site-specific, MAPK-dependent phosphorylation of Cx43 with subsequent alteration of NPC proliferation.

2.0 EXPERIMENTAL PROCEDURES

2.1 MOUSE NEURAL PROGENITOR CELL CULTURE

Mouse NPCs were prepared according to the technical manual provided by StemCell Technologies (StemCell Technologies, Vancouver, Canada). E14.5 embryos were obtained from pregnant C57Bl/6J (for Cav-1 KO) or CD1 mice. The Cav-1 KO animals were a generous gift from Dr. Ferruccio Galbiati (University of Pittsburgh, Department of Pharmacology, Pittsburgh, PA, USA). Pup brains were removed from the embryos, cortical lobes separated and the meninges removed. The tissue was disrupted by trituration and filtered through a 70µM mesh. $1x10^5$ cells/mL were plated on a 10cm Petri dish in 10mL of StemCell Technologies Proliferation Medium containing 20ng/mL recombinant human epidermal growth factor, 10ng/mL recombinant human fibroblast growth factor, and 2ug/mL heparin. Neurospheres that formed were passaged approximately every 4-5 days. For passaging, cells were collected by centrifugation for 5min at 500rpm, disassociated by pipetting 25X using a P200 pipette set at 100uL, and replated at $1x10^5$ cells/mL in fresh proliferation media containing all supplements. Cells were used between passages 2-6. The use of animals was approved and was in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh.

2.2 WESTERN BLOT ANALYSIS

20-100ug of total protein from cell lysates (collected in 10mM TRIS -pH 8.0, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 1% Triton X-100, 1mM PMSF, 0.1% Na-deoxycholate, and 0.1% SDS) were subject to SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane and probed with the appropriate primary antibody (Ab) and peroxidase conjugated secondary Ab. For isolation of Triton X-100 insoluble fractions, cells were lysed by a 30min incubation in 1% Triton X-100, 1mM EDTA and protease inhibitor in ice-cold Tris-buffered saline (TBS) followed by 30min centrifugation at 16.1 rcf. All blots were probed with primary Ab. at a 1:300 concentration and diluted in .1% BSA in PBST (.2% Tween) solution. The SNAP i.d. blotting system was used for all Western blots (Milipore, Temecula, CA). Primary Abs used for Western blot analysis include rabbit anti-Cx43, rabbit total ERK-1/2, mouse phospho-ERK-1/2 (Cell Signaling, Danvers, MA), mouse anti-Cx26 (Invitrogen, Carlsbad, CA), rabbit anti-phospho-Cx43 at serine 279/serine282, rabbit anti-phospho-Cx43 at serine 255, anti-GR (P20), anti-Cav-1 (N20) and goat anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary Abs were goat anti-mouse or anti rabbit HRP conjugate or a donkey anti-goat HRP conjugate (Bio-Rad, Hercules, CA). Secondary Abs were used at 1:1000 in .1% BSA in PBST. Proteins were visualized using a chemiluminescence visualization system (Perkinelmer, Shelton, CT). Images were quantified (densitometry) using NIH ImageJ software (ImageJ, http://rsbweb.nih.gov/ij/).

2.3 SUCROSE GRADIENT FRACTIONATION

NPC were washed 2X in ice cold phosphate-buffered saline (PBS) and placed in 2mL of a MES buffer (0.01M MES pH 6.5, 0.15M NaCl, and 1% (v/v) Triton X-100). Cells were homogenized using a loose fitting Dounce homogenizer and passing the glass tube 10X. The homogenate was adjusted to 40% sucrose by the addition of 2mL of 80% sucrose prepared in MES buffer and placed at the bottom of an ultracentrifuge tube. A 5-30% linear sucrose gradient was formed above the homogenate and centrifuged at 39,000 rpm at 4°C for 17hrs in a SW41 rotor (Beckman Instruments). Fractions were carefully removed following centrifugation, combined into caveolin-enriched fractions (#4-6) and non-caveolin enriched fractions (#9-11) and analyzed by SDS-PAGE and Western blots.

2.4 CO-IMMUNOPRECIPITATION

The entire protocol was carried out at 4°C. Cells were washed 2X in PBS and lysed in IP buffer containing 10mM Tris pH 8.0, 0.15 M NaCl, 5mM EDTA, 1% Triton X-100, 60mM octyl glucoside, and protease inhibitors. Samples were put on rotation for 45 minutes at 4°C. Soluble supernatant was precleared using protein A-Sepharose (10uL; slurry, 1:1) at 4°C. Samples were centrifuged at 13,000 rpm for 10min and supernatant was taken and normalized for protein concentration. One tenth of the volume was taken as an aliquot for total input. The supernatant was incubated overnight with the particular antibody of interest and protein A-Sepharose (30uL; slurry, 1:1). Beads were washed in lysis buffer 3 times on rotation for 10 minutes at 4°C. The final wash was done with 2.5mM of Tris-HCl pH 7.5. Beads were spun down at max speed for

1min and sample buffer was added and samples were boiled for 10min. The supernatant was then subjected to Western blot analysis for GR and Cav-1 as detailed above.

2.5 FRAP

Mouse NPCs between passages 2-6 on 35mm MatTek glass bottom culture dishes were treated with 1ug/mL of Calcein AM 30 min prior to FRAP analysis (Invitrogen, Carlsbad, CA). FRAP was conducted on an Olympus IX81 confocal microscope equipped with Fluoview data collection software. The photobleaching laser was set to 95% laser power for 1.8 seconds and recovery images captured every 40 seconds for 25 images. All data were quantified on open source NIH ImageJ software (ImageJ, http://rsbweb.nih.gov/ij/) and analyzed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA). FRAP recovery curves were fit to an exponential decay equation present in the GraphPad menu, and $t_{1/2}$ was tabulated by the software from this fit.

2.6 CO-IIF AND 5-BROMO-2'DEOXYURIDINE LABELING

10µM BrdU in 0.9% saline and 0.007M NaOH was added to NPCs 1hr prior to collection of cells. NPCs were collected by light centrifugation in a 15mL conical tube, fixed in 4% paraformaldehyde in PBS. BrdU epitopes were exposed by treatment of cells with 2N HCl, which also served to permeabilize cells. Cells were placed on glass coverslips for 30min in a 37°C incubator and allowed to settle onto the coverslip. Cells were then labeled with primary

and secondary antibodies using standard laboratory IIF conditions. The primary antibodies used were mouse anti-BrdU (1:500) (Sigma Aldrich, St Louis, MO) and rabbit anti-Ki-67 (1:100) (Abcam, Cambridge, MA). The secondary Abs included Alexa Fluor 488 goat anti-rabbit IgG (1:400) (Molecular Probes, Eugene, OR) or Cy3 conjugated goat anti-mouse IgG (1:400) (Jackson ImmunoResearch, West Grove, PA). Cells were visualized using an Olympus IX81 confocal microscope with Fluoview software (Olympus, Center Valley, PA).

2.7 STATISTICAL ANALYSIS

Statistical comparison was conducted by 1-way-analysis of variance (ANOVA). Post hoc Tukey HSD or Bonferroni was used to determine within group differences. A $p<0.05$ was considered statistically significant.

3.0 RESULTS

3.1 INHIBITION OF GJIC FOLLOWING A BRIEF EXPOSURE TO GC

NPCs from embryonic day 14.5 (E14.5) C57Bl/6 mice pups were utilized since they have the ability to differentiate into both glial and neuronal phenotypes *in vivo* and therefore provide a multipotent progenitor cell population. Furthermore, these NPCs are known to express Cx43-containing gap junctions (Viti et al. 2003; Cheng et al. 2004), which may play a role in coordinating their synchronous passage through the cell cycle (Weissman et al. 2004). NPCs were used between passages 2 and 10 to ensure enrichment of the NPC population (Jensen and Parmar 2006). To determine functional effects of GCs on gap junctions, NPCs were subjected to a 1hr treatment with the synthetic GR agonist dexamethasone (DEX), a cell impermeable DEX conjugate (i.e. DEX-BSA), and/or the GR antagonist RU-486. GJIC was quantified using a fluorescence recovery after photobleaching (FRAP) assay. FRAP (or gap-FRAP) is a well documented means of measuring GJIC that has high temporal resolution, is non-invasive, and uniquely, allows for precise determination of GJIC kinetics (Abbaci et al. 2008). The use of carbenexolone (Cbx), a gap junction inhibitor, confirmed that fluorescence recovery was specifically assessing GJIC.

A 1hr exposure of NPCs to 100nM DEX resulted in a significant increase in the $t_{1/2}$ of fluorescence recovery (Fig. 7 & Fig 8), which reflects an inhibition of GJIC. Co-treatment with

1µM of the GR antagonist RU-486 prevented the DEX mediated reduction in GJIC (Fig 7 and 8) indicating that the rapid inhibitory effect of GCs on GJIC in NPCs is GR dependent. All $t_{1/2}$ values were calculated by fitting a decaying exponential to the FRAP recovery curves (Fig. 7).

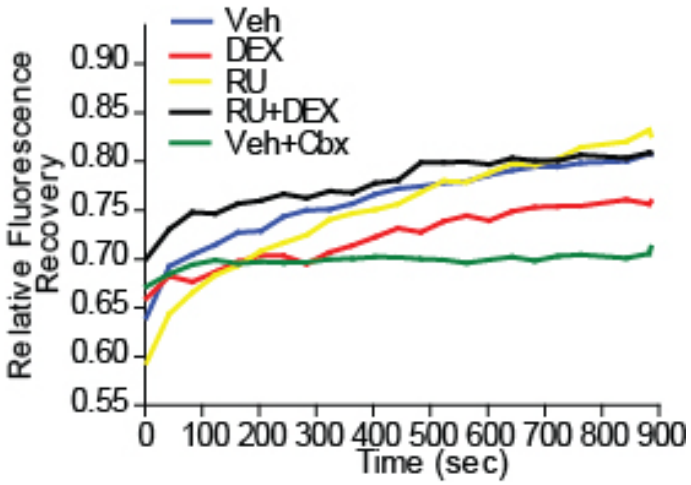

Figure 7. FRAP: Fluorescence Recovery Curves for 1hr DEX Treatment

NPCs preloaded with Calcein AM were subjected to the following 1 hr treatments; ethanol vehicle (Veh), 100nM DEX (+/- 1µM RU-486), RU-486 alone, and 200µM carbenoxolone (Cbx). Fluorescence recovery within individual bleached cells in a representative experiment is shown in Fig 7.

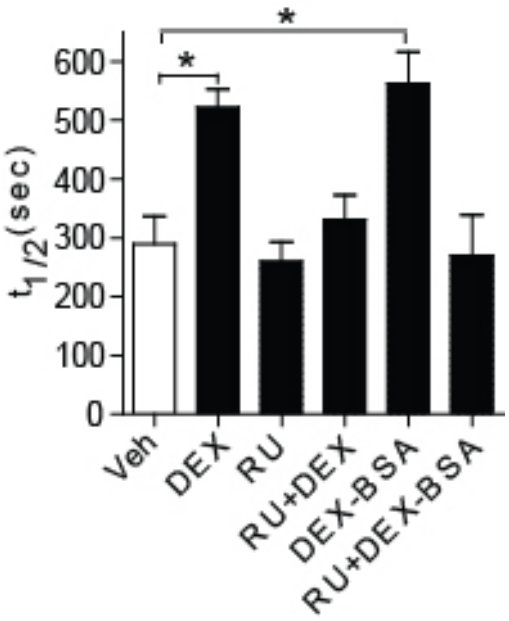

Figure 8: 1hr DEX Exposure Results in a Significant Increase in $t_{1/2}$

NPCs preloaded with Calcein AM were subjected to the following 1 hr treatments; ethanol vehicle (Veh), 100nM DEX (+/- 1μM RU-486), RU-486 alone, and 100nM DEX-BSA (+/- RU-486). Mean values for $t_{1/2}$ +SEM of recovery, are shown in Fig. 8. (*n*=4 independent experiments; 1-way ANOVA, $p<0.0001$; post hoc Tukey's Multiple Comparison Test, *=$p<0.05$).

The relatively rapid onset of the hormone effect prompted us to consider whether DEX-dependent inhibition of GJIC was mediated by non-classical GR action. Since most GR-dependent non-classical signaling mechanisms originate from activation of plasma membrane GR, a cell impermeable bovine serum albumin conjugated DEX (DEX-BSA) was utilized (Haller et al. 2008). Interestingly, a 1hr exposure of NPCs to 100nM DEX-BSA led to a significant loss in GJIC that was comparable to that observed with DEX exposure. In addition, co-treatment with 1μM RU-486 prevented the DEX-BSA mediated inhibition of GJIC (Fig 8). In combination, these results suggest that a GR-dependent non-classical signaling mechanism contributes to GC inhibition of GJIC in NPCs.

By measuring the fluorescence loss in all cells adjacent to the photobleached NPC, the number of NPCs connected to the photobleached NPCs can be tabulated. The $t_{1/2}$ values in Fig 8 were normalized to this number (Fig 9). Some photobleached NPCs were not connected to any other NPCs via gap junctions (Fig. 9) and did not show fluorescence recovery. The average number of cells connected to NPCs that recovered from photobleaching did not differ significantly between vehicle and hormone treated groups (Fig. 10).

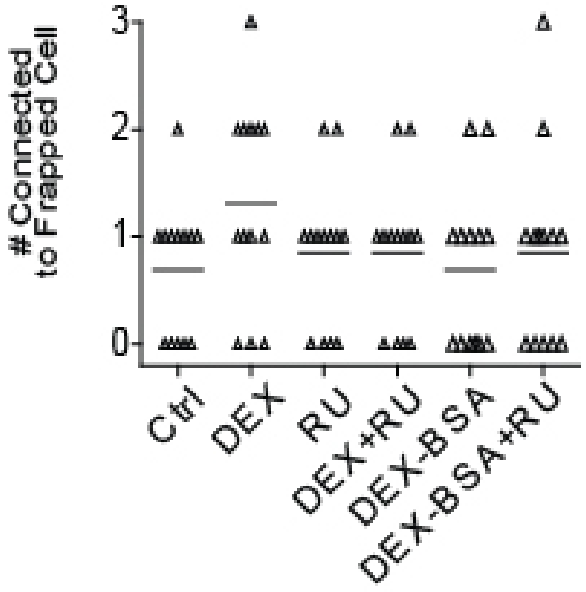

Figure 9: Number of NPCs Connected to NPCs Subject to FRAP

NPCs were prepared from E14.5 mouse embryonic cortices. Cultures preloaded with 1ng/µLCalcein AM for 15min were exposed to 100nM DEX or 100nM DEX-BSA +/- 1µM RU-486 where indicated for 1hr and GJIC measured using a gap-FRAP assay. In order to determine the number of adjacent NPCs connected to the NPC subject to FRAP, fluorescence loss in adjacent NPCs was simultaneously measured together with the gain in fluorescence in the photobleached (i.e., subject to FRAP) NPC. A loss in fluorescence in an adjacent NPC as the fluorescence increased in the photobleached NPC indicated a gap-junction mediated connection between the individual NPCs. Resulting $t_{1/2}$ values for fluorescence recovery were normalized to this value. For all NPCs subject to FRAP, there were between 0-3 connected NPCs.

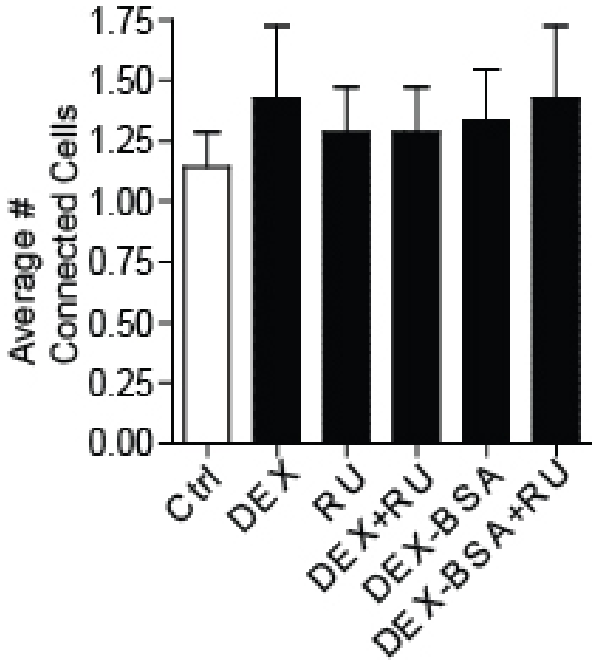

Figure 10: Average Number of NPCs Connected to FRAPPED NPC

Among those NPCs that showed fluorescence recovery, there were no significant differences in the average number of connected NPCs

3.2 BRIEF GC EXPOSURE LEADS TO RAPID PHOSPHORYLATION OF CX43

A reduction in GJIC may result from post-translational modifications (e.g. phosphorylation) of connexins that constitute gap junctions and/or from a change in connexin gene expression (Moreno and Lau 2007; Solan and Lampe 2009). In fact, GC has been shown to induce connexin-26 (Cx26) expression in cultured rat hepatocytes (Kojima et al. 1995). However, Western blot analysis failed to reveal effects of short term DEX exposure in NPC cultures on expression of total Cx26 and Cx43, two of the major connexin subtypes expressed in developing neuronal cells (Fig 11, 12, and 13). Since the activity of Cx43 in gap junctions, but not Cx26, is regulated by its phosphorylation at multiple sites, Western blot analysis was used to examine

DEX effects on overall Cx43 phosphorylation. As shown in Figures 12 and 14, a 1hr DEX treatment of NPCs led to an increase in expression of the slower migrating phosphorylated forms of Cx43 (i.e. P1 and P2) (Solan and Lampe 2009). In accordance with the results of gap-FRAP experiments (Fig 8 and 9), the DEX mediated increase in overall Cx43 phosphorylation was prevented by co-treatment with RU-486 and is therefore GR dependent. Furthermore, Triton fractionation experiments examining the proportion of membrane Cx43 and non-membrane Cx43 did not reveal any significant differences following a 1hr DEX treatment (Fig 15 and 16), indicating that changes in membrane localization of Cx43 do not account for the reduction in GJIC.

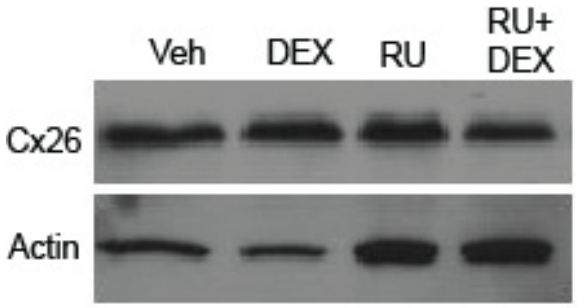

Figure 11: Cx26 Protein Levels Are Not Altered by 1hr DEX Exposure

NPC total protein lysates subjected to the following 1 hr treatments; ethanol vehicle (Veh), 100nM DEX (+/- 1µM RU-486), and RU-486 alone were analyzed by Western blot to determine Cx26 protein expression. Stripped blots were probed with an anti-actin antibody. Fig. 11 is a single representative blot (*n*=3).

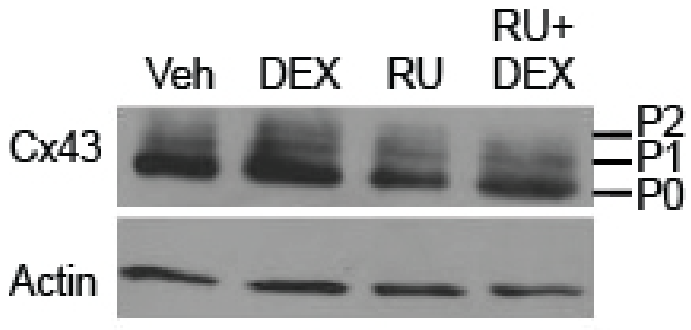

Figure 12: Cx43 Protein Expression Following a 1hr DEX Exposure

NPC total protein lyates from cells treated for 1 hr with ethanol vehicle (Veh), 100nM DEX (+/- 1µM RU-486), and RU-486 alone were subject to Western blot to determine Cx43 protein expression. Stripped blots were probed with an anti-actin antibody. P0, P1, and P2 in Fig 12 indicate positions of unphosphorylated (P0) and phosphorylated forms (P1, P2) of Cx43. Fig. 12 is a single representative blot (*n*=4).

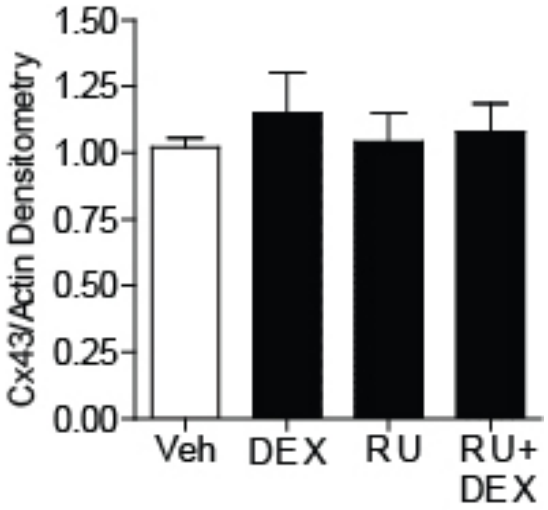

Figure 13: Total Cx43 Levels Are Not Altered by 1hr DEX Exposure

Fig 13 displays the mean +SEM of densitometric scans of multiple blots probed for total Cx43 and normalized to Actin following a 1hr DEX exposure (*n*=4).

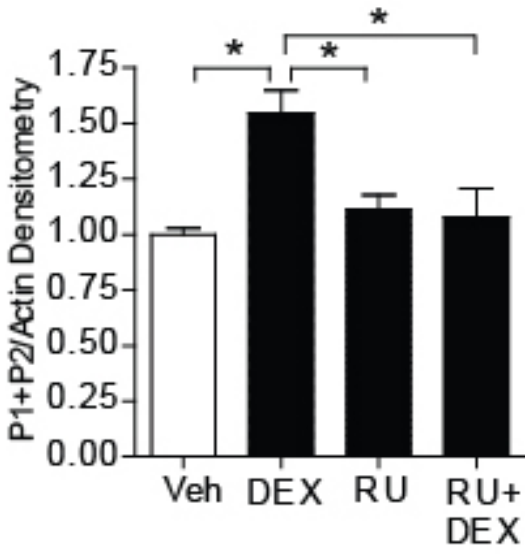

Figure 14: Cx43 Phosphorylation is Increased Following a 1hr DEX Exposure

Fig 14 displays the mean +SEM of densitometric scans of multiple blots probed for phosphorylated Cx43 (P1+P2) and normalized to Actin following a 1hr DEX exposure (*n*=4: 1-way ANOVA, p=0.0042; post hoc Tukey's Multiple Comparison Test, *=p<0.05).

Figure 15: Triton Fractionation of Cx43

NPCs prepared from E14.5 mouse embryonic cortices were treated for 1hr with ethanol vehicle (Veh) 100nM DEX and/or 1μM RU-486, where indicated. Cells were lysed in 1% Triton X-100 and PBS and separated into Triton insoluble (membrane) and soluble (cytosolic) fractions via high-speed centrifugation. Cell lysates were subjected to SDS-PAGE and Western blot analysis for total Cx43 expression. Fig 15 is a representative blot. (*n*=3).

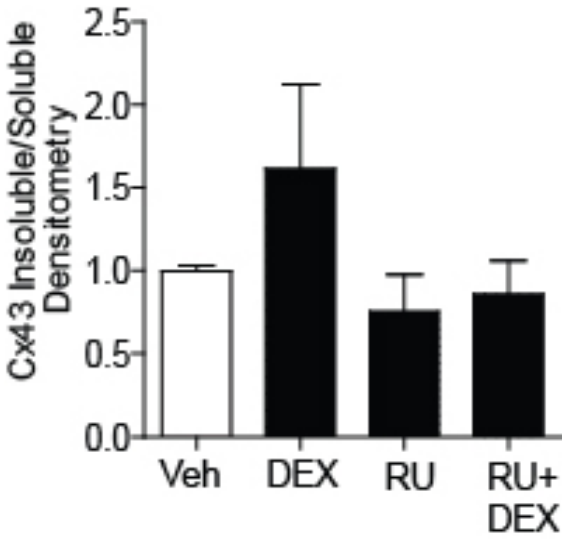

Figure 16: 1hr DEX Exposure Does Not Alter Cx43 Membrane Localization

Fig 16 displays the mean +SEM of the ratio of Cx43 soluble/insoluble fractions of densitometric scans of multiple blots. The bars represent Cx43 insoluble/Cx43 soluble fractions following 1hr DEX exposure. A 1hr DEX exposure had no effect on the ratio of Cx43 localized to the soluble vs. insoluble cell fraction. (*n*=3).

3.3 BRIEF GC EXPOSURE LEADS TO RAPID PHOSPHORYLATION OF ERK-1/2

Cx43 activity is differentially regulated by a variety of kinases. For example, phosphorylation of Cx43 at serines 279 and 282 by the mitogen-activated protein kinase (MAPK), extracellular signaling kinase-1/2 (ERK-1/2), leads to an inhibition of GJIC (Solan and Lampe 2009). MAPKs have been previously implicated in rapid GR and ER non-classical signaling (Qiu et al. 2001; Cato et al. 2002; Moriarty et al. 2006). A 1hr DEX exposure of NPCs led to a GR-dependent activation of ERK-1/2, as measured by Western blot analysis using a phospho-specific ERK-1/2 antibody (Fig 17 and 18). A detailed time-course of ERK-1/2 activation following DEX treatment revealed a rapid and biphasic increase in pERK-1/2. Specifically, pERK-1/2 is significantly increased 2min following DEX treatment then decreases to baseline at 15 and

30min, but is significantly increased again following a 1hr DEX treatment (Fig 19 and 20). Importantly, ERK-1/2 remains activated following an exposure to DEX (i.e. at 1hr), which we have shown triggers increased overall Cx43 phosphorylation and reduced GJIC.

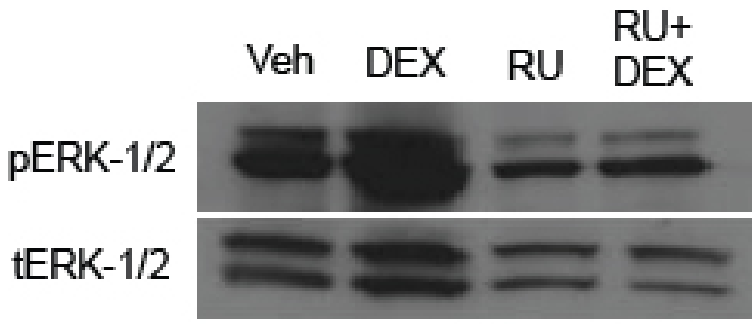

Figure 17: pERK-1/2 Protein Levels Following 1hr DEX Exposure

Total protein lysates from NPCs treated for 1hr with 100nM DEX and/or 1μM RU-486 were subjected to Western blot analysis to assess phosphorylated-ERK-1/2 (pERK-1/2) and total ERK-1/2 (tERK-1/2). Fig 17 is a representative Western blot. (*n*=4).

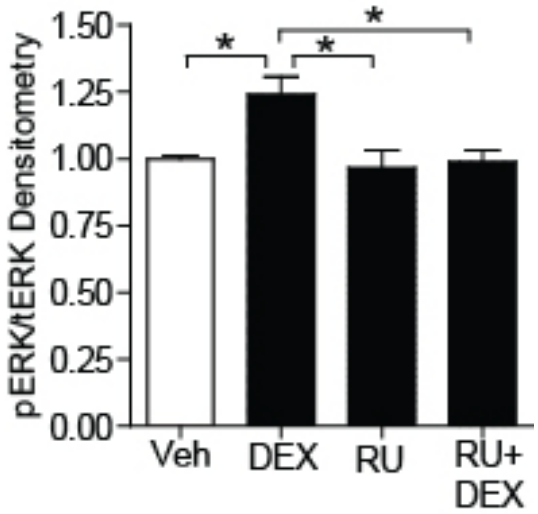

Figure 18: 1hr DEX Exposure Leads to a Significant Increase in pERK-1/2 Levels

Fig 18 is a densitometric scan of multiple blots. The bars represent the ratio of pERK/tERK following 1hr DEX exposure. Significant effects of DEX treatment on pERK-1/2 levels are observed (*n*=4; 1-way ANOVA, P=0.0085; post hoc Tukey's Multiple Comparison Test, *=P<0.05).

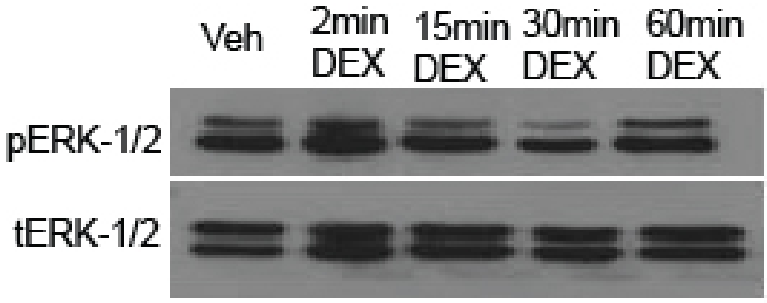

Figure 19: Time Course of pERK-1/2 Protein Levels Following DEX Exposure

Total protein lysates from NPCs treated for various times between 2min and 1hr with 100nM DEX. Lysates were subjected to Western blot analysis to assess phosphorylated-ERK-1/2 (pERK-1/2) and total ERK-1/2 (tERK-1/2). Image is a representative Western blot. (*n*=5).

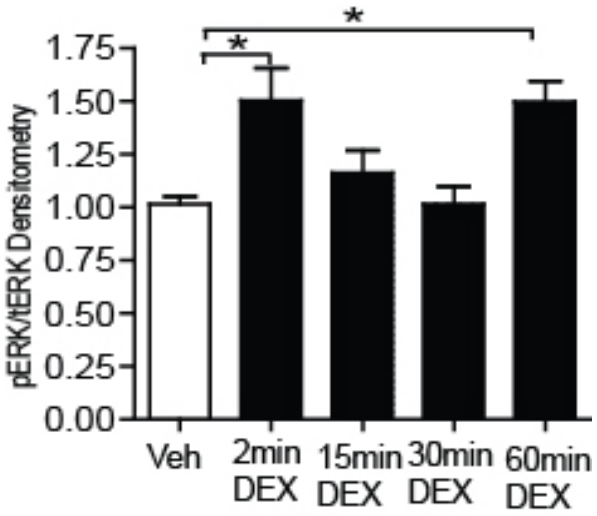

Figure 20: DEX Exposure Leads to a Rapid and Biphasic Increase in pERK-1/2 Levels

Fig 20 represents a densitometric scan from multiple blots of pERK-1/2/ERK-1/2 following DEX exposure. Significant effects of DEX treatment on pERK-1/2 levels were observed at 2min and 60min of DEX exposure (*n*=4; 1-way ANOVA, P=0.0050; post hoc Tukey's Multiple Comparison Test, *=P<0.05).

3.4 GC EXPOSURE LEADS TO RAPID, SITE SPECIFIC, PHOSPHORYLATION OF CX43

In order to determine if Cx43 phosphorylation at the ERK-1/2 target sites occurs in response to GC exposure, Western blot analysis was performed using an antibody directed against Cx43 phosphorylated at serines 279 and 282 (pCx43s279s282) and using an antibody against Cx43 phosphorylated at serine 255 (pCx43s255). As shown in Figures 21 and 22, a 1hr DEX exposure of NPCs led to a significant increase in pCx43s279s282. This increase was not present in NPCs treated with DEX and RU-486 (Fig. 21 and 22). Interestingly, as shown in figures 23 and 24 DEX exposure did not lead to a significant effect on pCx43s255.

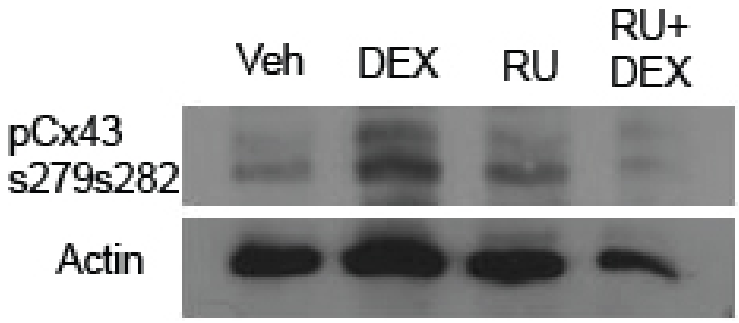

Figure 21: pCx43s279s282 Protein Levels Following 1hr DEX Exposure

Total protein lysates from NPCs treated for 1hr with ethanol vehicle (Veh), 100nM DEX and/or 1µM RU-486 were subjected to Western blot analysis to assess phosphorylated connexin 43 at serine 279 and 282 (pCx43s279s282) and Actin. A representative Western blot is shown in Fig 21. (*n*=4).

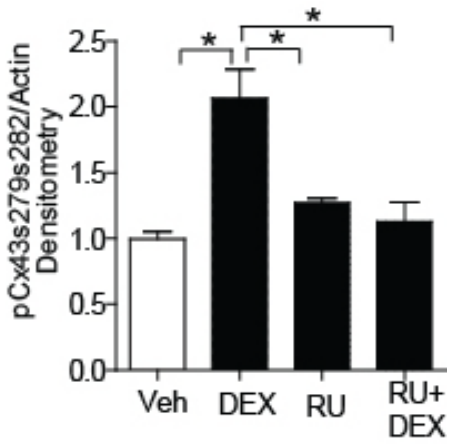

Figure 22: 1hr DEX Exposure Leads to a Significant Increase in pCx43s279s282 Levels

Fig 22 represents a densitometric scan from multiple blots of pCx43s279s282 following a 1hr DEX exposure. Significant effects of DEX treatment on pCx43s279s282 were observed (*n*=4; 1-way ANOVA, P=0.0023; post hoc Tukey's Multiple Comparison Test, *=P<0.05).

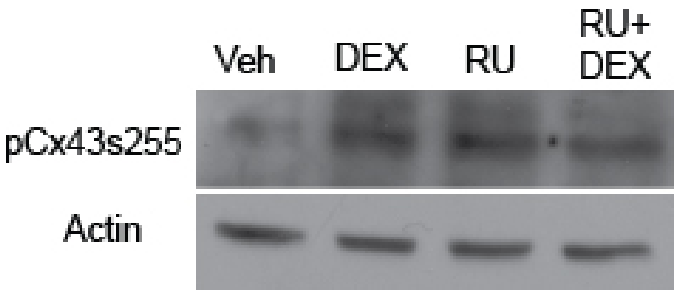

Figure 23: pCx43s255 Protein Levels Following 1hr DEX Exposure

Total protein lysates from NPCs treated for 1hr with ethanol vehicle (Veh), 100nM DEX, and/or 1µM RU-486 were subjected to Western blot analysis to assess phosphorylated connexin 43 at serine 255 (pCx43s255) and Actin. A representative Western blot is shown in Fig 23. (*n*=4).

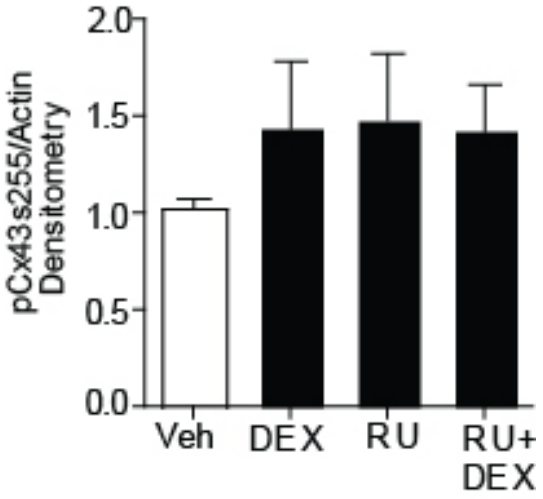

Figure 24: 1hr DEX Exposure Does Not Alter pCx43s255 Levels

Fig 24 represents a densitometric scan from multiple blots of pCx43s255 following a 1hr DEX exposure. Significant effects of DEX treatment on pCx43s255 were not observed. (*n*=4).

3.5 GC INDUCED CX43 PHOSPHORYLATION AND REDUCTION OF GJIC ARE ERK-1/2 DEPENDENT

In order to determine if GR effects on Cx43 phosphorylation and function are dependent on ERK-1/2, Western blot analysis of pCx43s279s282 and FRAP were performed in the presence of the MEK-1/2 inhibitor PD98059 (PD). A 40µM dose of PD inhibits DEX mediated ERK-1/2 activation (Fig. 25). As shown in Figures 26 and 27, a 40µM PD co-treatment of NPCs prevented the increase in pCx43s279s282 following 1hr DEX exposure. In addition, 40µM PD treatment also prevented the DEX-mediated decrease in GJIC (Fig. 28). Therefore, ERK-1/2 activation appears necessary for phosphorylation of Cx43 at serine 279/282 and for the DEX-mediated inhibition of GJIC.

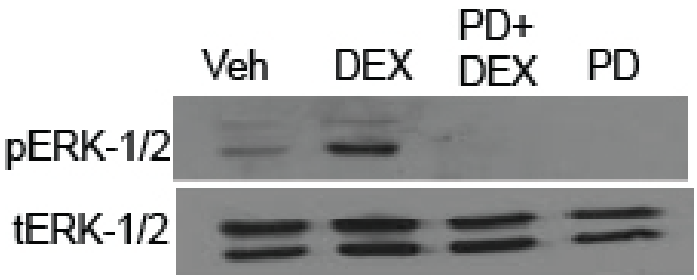

Figure 25: pERK-1/2 Protein Levels Following 1hr DEX and PD Exposure

Western blot of total protein lysates from NPCs treated for 1hr with ethanol vehicle (Veh), 100nM DEX, and/or 40µM PD98059. Lysates were subject to Western blot analysis to measure levels of pERK-1/2 and tERK-1/2. (*n*=1).

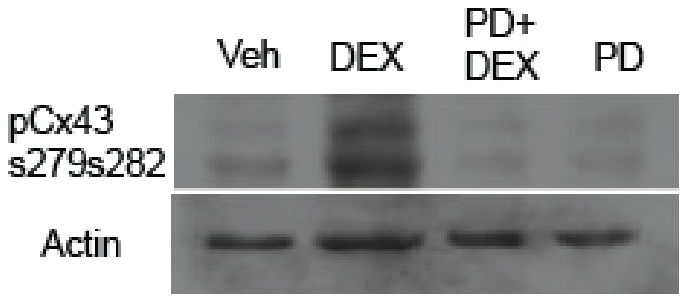

Figure 26: pCx43s279s282 Protein Levels Following 1hr DEX and PD Exposure

Total protein lysates from NPCs treated for 1hr with ethanol vehicle (Veh), 100nM DEX, and/or 40µM PD98059 were subject to Western blot analysis to measure levels of pCx43s279s282 and Actin. Fig. 26 is an image of a representative Western blot. (*n*=4).

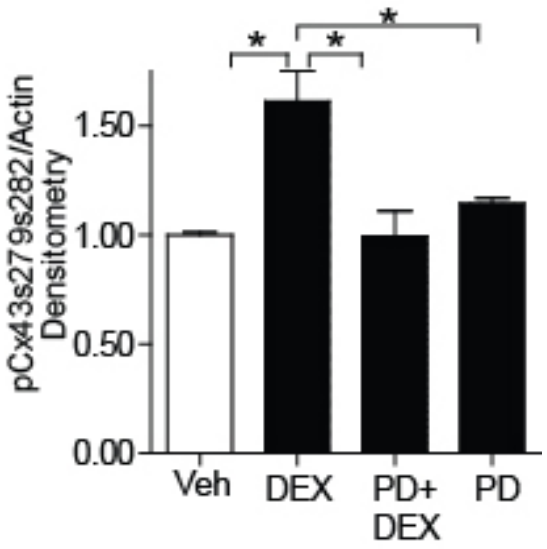

Figure 27: PD Co-Tx Inhibits the Increase of pCx43s279s282 Following 1hr DEX Exposure

Fig. 27 displays results of the mean + SEM of densitometric scans of multiple Western blots from NPC lysates subject to 1hr DEX and/or PD treatment. The DEX induced increase in PD is attenuated by PD co-treatment (*n*=4; 1-way ANOVA, P=0.0012; post hoc Tukey's Multiple Comparison Test, *=P<0.05).

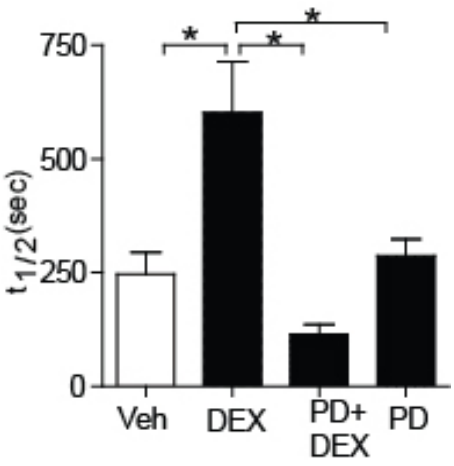

Figure 28: PD Co-Tx Inhibits the Decrease in GJIC Induced by 1hr DEX Exposure

NPCs preloaded with Calcein AM were subjected to ethanol vehicle (Veh) or 100nM DEX +/- 40μM PD followed by FRAP analysis to measure GJIC. Mean values for $t_{1/2}$ +SEM of recovery are shown in Fig 28. PD co-treatment prevented the DEX mediated reduction in GJIC (*n*=5 independent experiments,1-way ANOVA, P=0.0002; post hoc Tukey's Multiple Comparison Test, *= P<0.05).

3.6 *DENOVO* GENE TRANSCRIPTION IS NOT NECESSARY FOR GC EFFECTS ON CX43 PHOSPHORYLATION OR GC INHIBITION OF GJIC

In order to further corroborate the pathway of non-classical GR effects on GJIC, DEX-exposed NPCs were subjected to a 1hr pretreatment with 100ng/mL of the transcriptional inhibitor Actinomycin D (ActD). Quantitative RT-PCR (qRT-PCR) indicated that 1hr ActD pretreatment effectively inhibited DEX induction of the GC responsive gene glucocorticoid-induced leucine zipper (*GILZ*) (Fig. 29). Western blot analysis indicates that ActD pretreatment had no effect on the induction of pCx43s279s282 following 1hr DEX treatment (Fig. 30 and 31). Similarly, gap-FRAP experiments reveal that ActD pretreatment had no effect on inhibition of GJIC following 1hr DEX exposure (Fig. 32). These results, along with those from previous studies with DEX-BSA (Fig. 9) support the notion that a non-classical transcription-independent mechanism underlies GC effects on GJIC in NPC cultures.

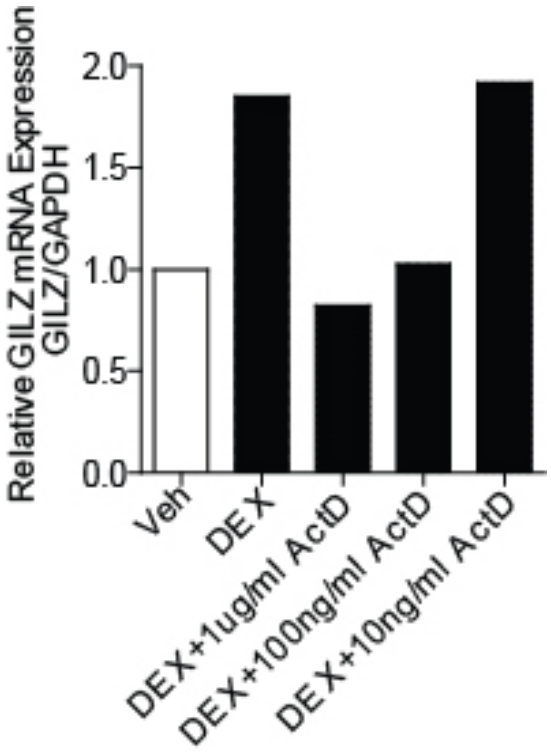

Figure 29: 100ng/mL ActD Pretreatment Prevents DEX Induction of a GR Target Gene

NPCs prepared from E14.5 mouse embryonic cortices were pretreated for 1hr with various concentrations of the transcriptional inhibitor Actinomycin D (ActD) followed by a 1hr exposure to 100nM DEX. Cells were lysed in Trizol, RNA was extracted, and cDNA was prepared from the RNA template. Expression of the GR target gene glucocorticoid induced leucine zipper (GILZ) relative to the house-keeping gene GAPDH was measured by qRT-PCR. 100ng/mL of ActD pretreatment was sufficient to prevent DEX induced GILZ expression. (*n*=1).

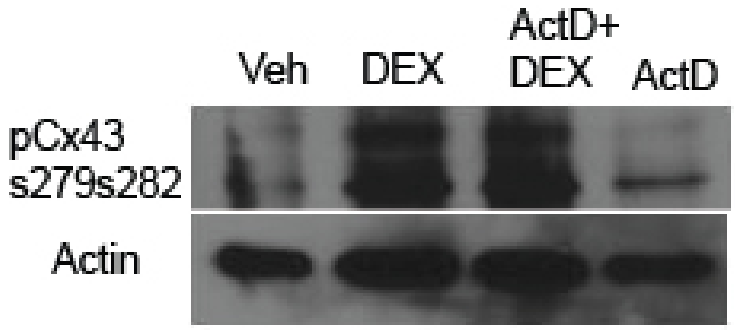

Figure 30: pCx43s279s282 Protein Levels Following 1hr ActD Pretreatment and 1hr DEX Exposure

Western blot of total protein lysates from NPCs pretreated with 100ng/mL ActD prior to a 1hr 100nM DEX treatment. Ethanol vehicle (Veh) was used as a control. Lysates were subject to Western blot for pCx43s279s282 and Actin. Fig 30 is a representative blot. (*n*=4).

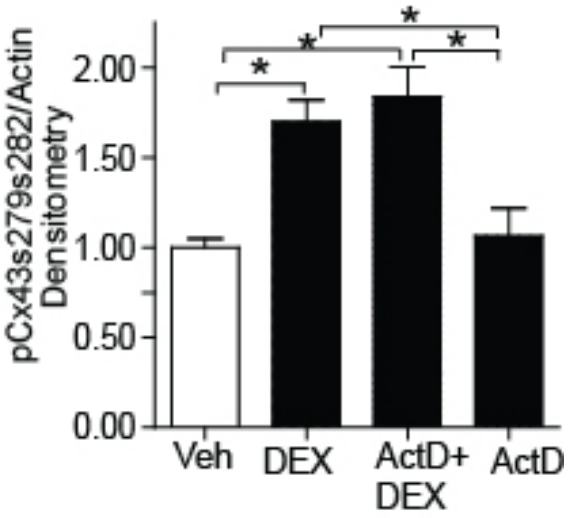

Figure 31: ActD Pretreatment Does Not Inhibit Cx43 Phosphorylation at s279s282 Following a 1hr DEX Exposure.

Fig. 31 displays results of the mean + SEM of densitometric scans of multiple Western blots from NPC lysates subject to 1hr ActD pretreatment followed by a 1hr DEX exposure. Transcriptional inhibition by ActD did not alter DEX induced Cx43 phosphorylation at s279s282 (*n*=4; 1-way ANOVA, P=0.0009; post hoc Tukey's Multiple Comparison Test, *=P<0.05).

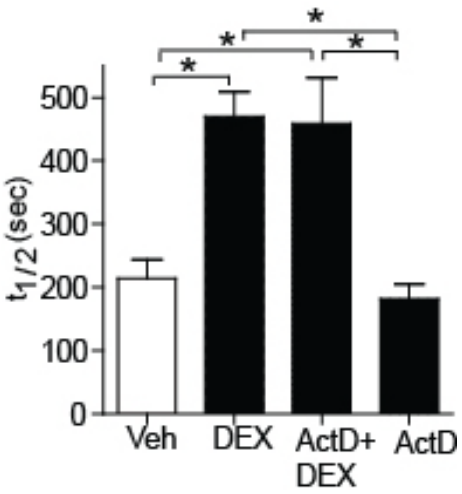

Figure 32: ActD Pretreatment Does Not Alter the Reduction in GJIC Following a 1hr DEX Exposure.

NPCs preloaded with Calcein AM were subjected to 100ng/mL ActD pretreatment +/- 100nM DEX followed by FRAP analysis to measure GJIC. Ethanol vehicle (Veh) was used as a control. Mean values for $t_{1/2}$ +SEM of recovery are shown in Fig. 32. ActD pretreatment had no effect on the DEX mediated reduction in GJIC (*n*=4 independent experiments, 1-way ANOVA, P <0.0001; post hoc Tukey's Multiple Comparison Test, *=P<0.05)

3.7 GR IS ASSOCIATED WITH CAVEOLIN-1 IN LIPID RAFTS OF NPCS

Caveolae are specialized membrane invaginations localized to sphingolipid-rich domains called lipid rafts (Langlois et al. 2008). Caveolin-1 (Cav-1) is a major protein component of caveolae and has been implicated in membrane GR signaling and in facilitating Cx43 dependent GJIC (Langlois et al. 2008; Matthews et al. 2008). Specifically, Cav-1 has been found to co-immunoprecipitate with Cx43 and overexpression of Cav-1 has been shown to increase GJIC (Matthews et al. 2008). Therefore, two independent biochemical analyses were performed to reveal whether GR is associated with Cav-1 in NPCs. Western blot analysis of sucrose gradient fractions revealed expression of GR in the Cav-1 enriched membrane fraction (i.e. Fractions 4-6; Fig. 33). GR expression in the Cav-1 enriched fraction remains relatively constant following 1hr

DEX exposure (Fig. 33). As an independent assessment of GR/Cav-1 interactions, co-immunoprecipitation experiments were performed with whole cell-free lysates from NPC cultures. As shown by reciprocal co-immunoprecipitation experiments in Figures 34 and 35, GR and Cav-1 exist in a complex that is not affected by a 1hr DEX exposure.

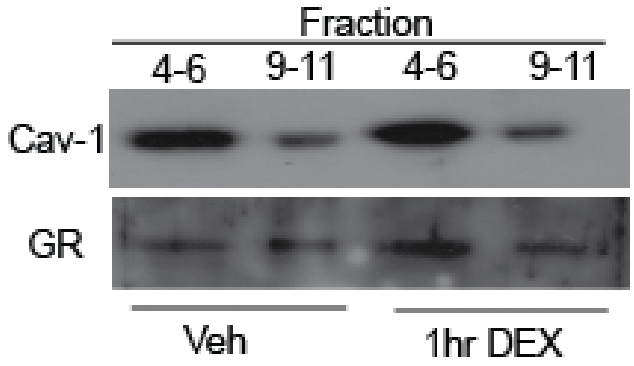

Figure 33: GR is Present in the Cav-1 Enriched Membrane Fraction

Ethanol vehicle (Veh) or 1hr 100nM DEX (1hr DEX) treated NPC extracts subjected to sucrose gradient fractionation to enrich for Cav-1 membrane fractions (i.e. fractions 4-6) were analyzed for GR and Cav-1 expression using Western blots. One representative blot is shown in Fig 33. (*n*=2).

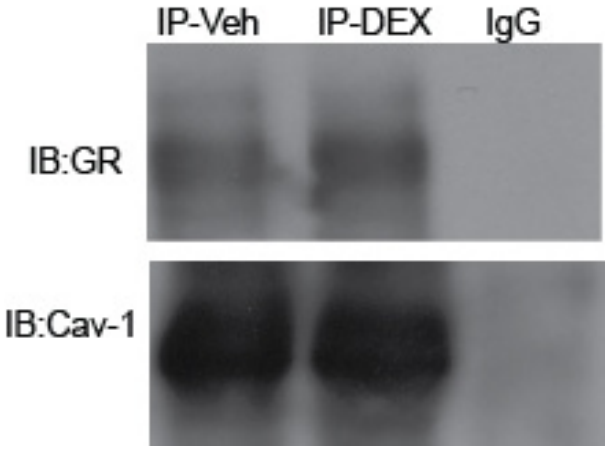

Figure 34: Cav-1 IP; GR Associates with Cav-1 in a DEX Independent Manner

Triton-soluble extracts were subjected to a co-immunoprecipitation assay with subsequent Western blot to reveal an association between GR and Cav-1. Extracts were subject to 1hr ethanol vehicle (IP-Veh) or 100nM DEX (IP-DEX) exposure. A non-immune IgG was used as a control co-immunoprecipitation. A representative blot is shown in Fig 34. (*n*=3).

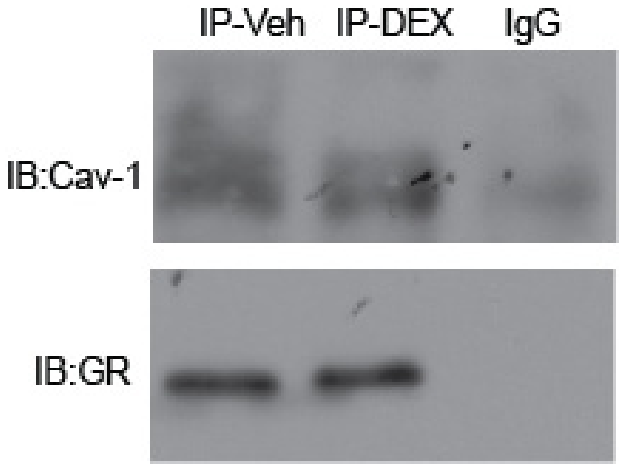

Figure 35: GR IP; Cav-1 Associates with GR in a DEX Independent Manner

Triton-soluble extracts were subjected to a co-immunoprecipitation assay with subsequent Western blot to reveal an association between GR and Cav-1 Extracts were subject to 1hr ethanol vehicle (IP-Veh) or 100nM DEX (IP-DEX) exposure. A non-immune IgG was used as a control co-immunoprecipation. A representative blot is shown in Fig 35. (*n*=3).

3.8 C-SRC INHIBITION PREVENTS GC ACTIVATION OF ERK-1/2

GR and the non-receptor tyrosine kinase c-src have been previously reported to localize to Cav-1 enriched membrane fractions. Furthermore, c-src was found to be a critical downstream signaling protein in a GR non-classical signaling mechanism (Matthews et al. 2008). In light of our findings revealing a GR/Cav-1 interaction (Fig 33-35), we examined the role of c-src on GC-induced ERK-1/2 activation. A 30min pretreatment of NPCs with 10μM of the src family inhibitor PP2 followed by a 1hr DEX exposure prevented the DEX-mediated increase in pERK-1/2 (Fig 36 and 37) suggesting that c-src activation is coupled to GR-dependent ERK-1/2 activation and Cx43 phosphorylation.

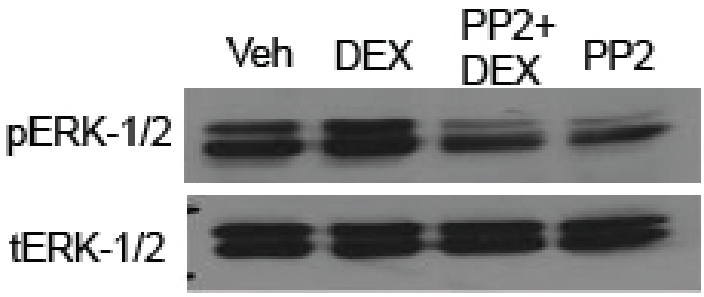

Figure 36: pERK-1/2 Protein Levels in PP2 Pretreated NPCs Subject to 1hr DEX Exposure

Total protein lysates from NPCs pretreated with 10µM PP2 for 30min followed by a 1hr exposure to 100nM DEX were subjected to Western blot analysis to measure pERK-1/2 levels. Fig 36 represents a representative blot. (*n*=6).

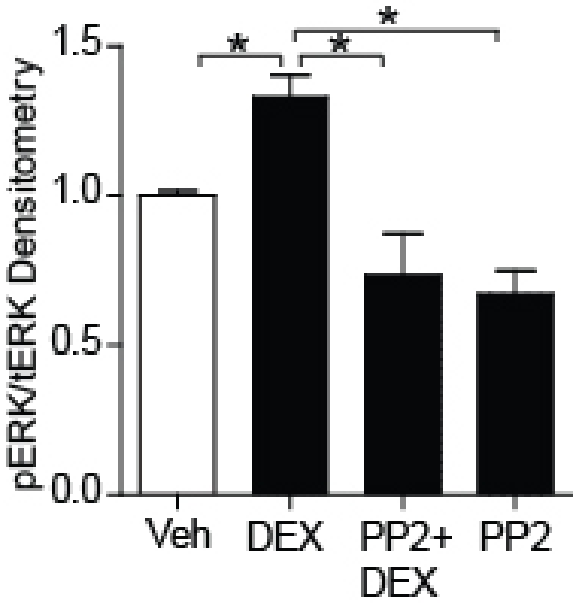

Figure 37: PP2 Pretreatment Inhibits ERK-1/2 Activation Following a 1hr DEX Exposure.

Fig 37 displays the results of the mean + SEM of densitometric scans of multiple Western blots from NPC lysates subject to PP2 pretreatment followed by a 1hr DEX exposure. Ethanol vehicle (Veh) was used as a control. Significant effects of DEX were revealed in results of the mean +SEM ratio of pERK/tERK from densitometric scans of multiple blots (*n*=6; 1-way ANOVA, P =0.0001; post hoc Bonferroni's Multiple Comparison Test, *=P<0.05).

3.9 TRANSIENT GC EXPOSURE IS SUFFICIENT TO REDUCE S PHASE PROGRESSION IN NPCS AND ENHANCE CELL CYCLE EXIT

GC inhibition of NPC proliferation observed *in-vitro* could underlie the detrimental effects of embryonic exposure to these hormones that has been observed in animal and clinical studies (Yeh et al. 2004; Yu et al.). While transcriptional targets of GR that influence cell cycle progression in NPCs have been identified, the contribution of non-classical GR signaling to NPC proliferation has not been addressed (Rogatsky et al. 1997; Ayroldi et al. 2007). Both classical and non-classical actions of estrogen receptor (ER) regulate breast cancer cell proliferation providing precedence for multi-level action of steroid receptors in cell cycle control (Razandi et al. 2004; Levin 2005).

A prolonged (i.e. 24hr) DEX treatment reduced NPC proliferation in these cultures (Fig 38-39), as observed in NPCs obtained from other brain regions and ages (Sundberg et al. 2006; Yu et al.). NPC proliferation in neurosphere cultures was not altered upon a simultaneous 24hr treatment with RU-486 and DEX, demonstrating the GR dependence of the anti-proliferative GC effects. Figure 38 and 39 display the two independent assays used to assess NPC proliferation in neurosphere cultures. A 1hr BrdU pulse immediately preceding cell harvest was used to identify cells progressing through S phase. NPCs positive for BrdU staining were revealed by indirect immunofluorescence (IIF) with an anti-BrdU antibody. IIF was also used to detect NPCs positive for Ki67, which is expressed in cells actively progressing through the cell cycle (i.e. G1, S, G2/M). Cells that have exited the cell cycle (i.e. G0) no longer express Ki67.

In order to limit the duration of GR activity, RU-486 was added to neurosphere cultures following a 1hr pre-exposure to DEX (preDEX+RU). NPC proliferation was then assessed 23hrs later using a 1hr BrdU pulse and Ki67 staining as described above. As shown in Figures 38 and

39, a 1hr DEX "pulse" was sufficient to generate an antiproliferative effect on NPCs as assessed by both BrdU and Ki67 staining. The reduction in BrdU incorporation and Ki67 staining in NPCs exposed chronically or transiently to DEX suggests that GCs are both limiting the entry of NPCs into S phase and enhancing cell cycle exit.

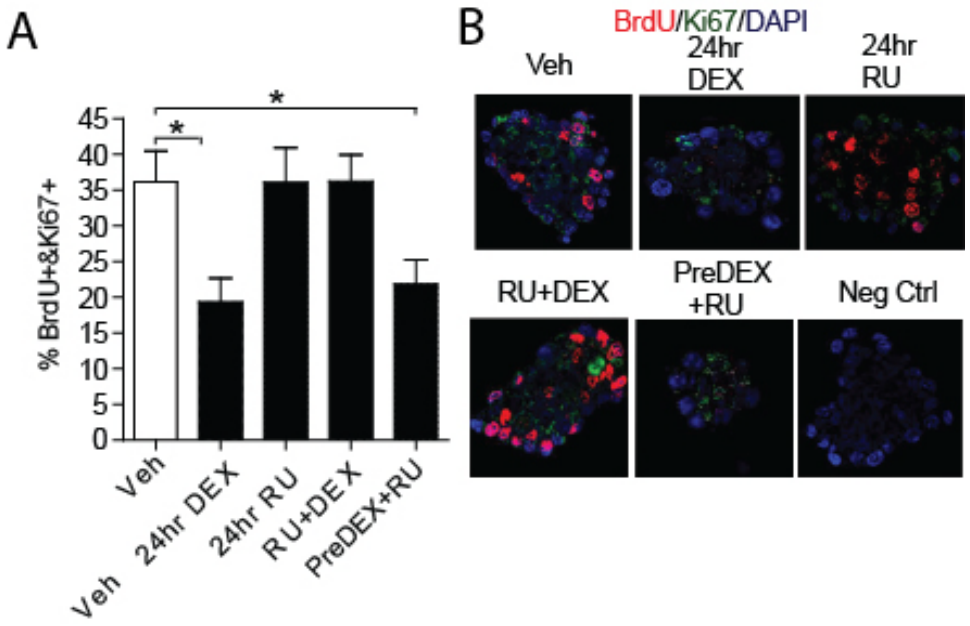

Figure 38: A 1hr DEX Exposure Reduces BrdU+ NPCs 24hrs Following Treatment

NPCs were subjected to the following 24 hr treatments; ethanol vehicle (Veh), 100nM DEX (+/- 1µM RU-486), RU-486 alone, or a 1hr DEX pretreatment followed by a 23 Hr RU-486 exposure (1hr PreDEX +RU). NPCs were treated with a 10µM BrdU pulse during the final hour of hormone exposure and then processed for immunostaining to detect BrdU incorporation and Ki67 expression. Mean values for BrdU+/Ki67+ cells +SEM in Fig 38 panel A show a significant reduction in NPCs actively in S-phase of the cell cycle (*n*=3; 4 random fields per image; 1-way ANOVA, P =0.0024; post hoc Tukey's Multiple Comparison Test, *=P<0.05). Panel B is a representative image.

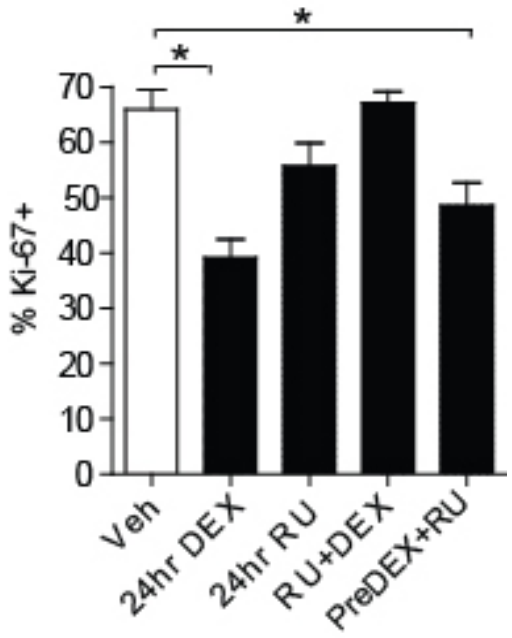

Figure 39: A 1hr DEX Exposure Reduces Ki67+ NPCs 24hrs Following Treatment

NPCs were subjected to the following 24 hr treatments; ethanol vehicle (Veh), 100nM DEX (+/- 1µM RU-486), RU-486 alone, or a 1hr DEX pretreatment followed by a 23 Hr RU-486 exposure (1hr PreDEX +RU). NPCs were treated with a 10µM BrdU pulse during the final hour of hormone exposure and then processed for immunostaining to detect BrdU incorporation and Ki67 expression. Analysis of Ki67 immunostained cells alone (Fig 39) indicated a significant reduction in NPCs actively engaged in any phase of the cell cycle (i.e. G1-S-G2/M) (n=4; 4 random fields per image; 1-way ANOVA, $P < 0.0001$; post hoc Tukey's Multiple Comparison Test, *= $P<0.05$).

3.10 TRANSIENT INHIBITION OF GJIC IS SUFFICIENT TO REDUCE S PHASE PROGRESSION IN NPCS BUT DOES NOT TRIGGER CELL CYCLE EXIT

While the 1hr DEX "pulse" used above may not necessarily limit GR to non-classical effects, this duration of GC exposure does initiate a non-classical and transcription-independent signaling pathway in NPCs. This culminates in reduced GJIC between connected cells (Fig 8). In order to examine whether a transient inhibition of GJIC (e.g. brought about by non-classical

GR signaling) could affect NPC proliferation, NPC proliferation assays were performed following exposure of neurosphere cultures to the reversible GJIC inhibitor 1-heptanol (Kimura et al. 1995). Treatment of neurosphere cultures with 3mM 1-heptanol led to a loss in GJIC in NPCs that was sustained for 1hr, but could be rapidly reversed following removal of 1-heptanol (Fig 40). NPC proliferation was then examined by exposing neurosphere cultures to 3mM 1-heptanol for only 1hr. Specifically, cultures were extensively washed in fresh media following this exposure and proliferation assessed 23hrs later by a 1hr BrdU pulse and subsequent IIF to detect BrdU and Ki67 positive cells. As shown in Figure 41, a 1hr 1-heptanol exposure led to a significant reduction in BrdU positive NPCs, but surprisingly, did not have any effect on the number of Ki67 positive cells (Fig 42). Therefore, while either transient GR activation or GJIC inhibition is sufficient to limit NPC entry into S phase, cell cycle exit most likely requires additional actions of GR that extend beyond its non-classical effects limiting GJIC.

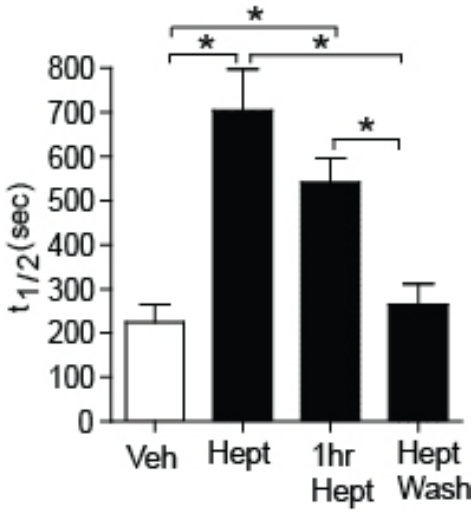

Figure 40: Heptanol Exposure Transiently Reduces GJIC in NPCs

NPCs preloaded with Calcein AM were subjected to the following treatments: Ethanol vehicle (Veh), 2 min 1-heptanol (Hept), 1hr Hept, and 1hr Hept followed by a wash into heptanol free media (Hept Wash). 3mM Hept was used in all treatments. Mean values for $t_{1/2}$ +SEM of recovery obtained by fitting a decayed exponential to individual fluorescence recovery curves show reversible inhibition of GJIC by Hept (*n*=4, 1-way ANOVA, P <0.0001; post hoc Tukey's Multiple Comparison Test, *=P<0.05).

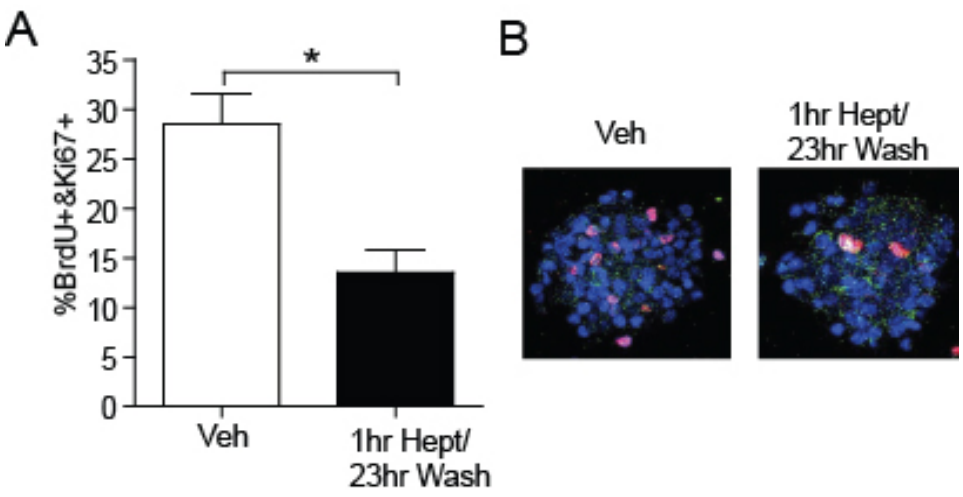

Figure 41: 1hr Hept Exposure Reduces BrdU+ NPCs 24hrs Following Treatment

The proliferation assay in Fig 41 Panel A was performed as described above (see Fig 39) following a 1hr 3mM heptanol exposure followed by washout. The results reveal a significant effect of a limited (i.e. 1 hr) heptanol exposure on NPCs actively progressing through S-phase of the cell cycle measured 23 Hrs following heptanol removal and wash (1hr Hept/23hrWash). (Fig 41 panel A: mean number +SEM of BrdU+/Ki67+ cells, *n*=4; 4 random fields per image; 1-way ANOVA, P <0.0001; post hoc Tukey's Multiple Comparison Test, *=P<0.05). Fig 41 Panel B is a representative image.

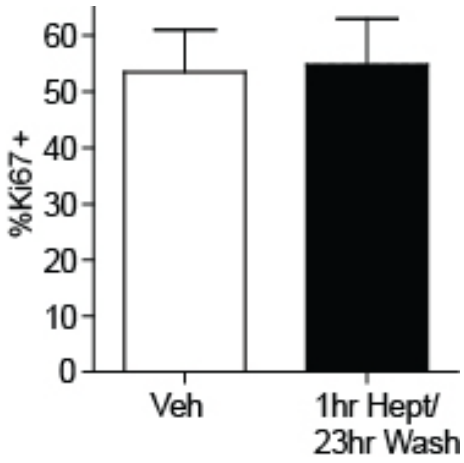

Figure 42: 1hr Hept Exposure Has No Effect on Ki67+ NPCs 24hrs Following Treatment

The proliferation assay in Fig 42 was performed as described above (see Fig 39) following a 1hr heptanol exposure followed by washout. Analysis of mean +SEM of Ki67 only labeled cells (Fig 42) reveals no significant effect of the limited heptanol exposure on NPCs exiting the cell cycle. (*n*=4).

3.11 CAV-1 IS NECESSARY FOR RAPID GC MEDIATED ERK-1/2 PHOSPHORYLATION, CX43 PHOSPORYLATION, AND REDUCTION OF GJIC

In order to definitively assess the role of cav-1 in the signaling cascade that leads to GC mediated Cx43 phosphorylation and a reduction in GJIC, experiments were performed on NPCs derived from Cav-1 knockout (Cav-1 KO) animals. Exposure of Cav-1 KO NPCs to 1hr of DEX did not lead to a significant change in pERK-1/2 levels as measured by Western blot (Fig 43 and Fig 44). A 1hr DEX exposure of Cav-1 KO NPCs also did not have any significant effects on pCx43s279s282 (Fig 45 and Fig 46). In order to determine the functional effect of Cav-1 KO on GJIC, FRAP experiments were performed as described previously. No significant effects on GJIC were observed from a 1hr DEX exposure of Cav-1 KO NPCs (Fig 47). In

combination, these results indicate that cav-1 is essential for rapid GC mediated signaling that results in ERK-1/2 mediated Cx43 phosphorylation at s279/s282 and subsequent reductions in NPC GJIC.

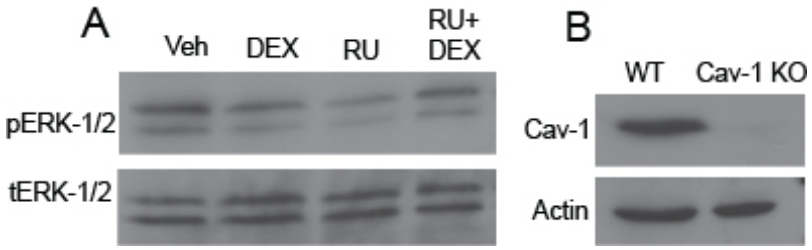

Figure 43: pERK-1/2 Levels in Cav-1 KO NPCs Following 1hr DEX Exposure

Total protein lysates from Cav-1 KO NPCs treated for 1hr with ethanol vehicle, 100nM DEX, and/or 1μM RU-486 were subjected to Western blot analysis to assess phosphorylated-ERK-1/2 (pERK-1/2) and total ERK-1/2 (tERK-1/2). Fig 43 panel A is a representative Western blot. (*n*=3). Fig 43 Panel B displays total lysates from Cav-1 KO and WT NPCs subject to Western blot analysis to assess Cav-1 and Actin. (*n*=1).

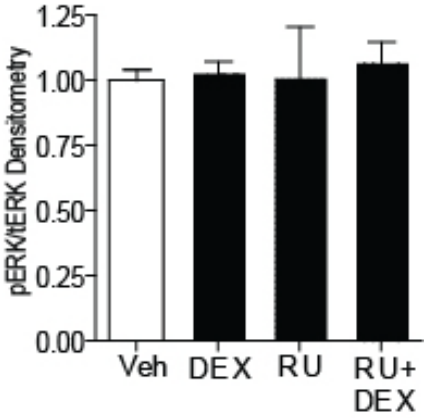

Figure 44: 1hr DEX Exposure of Cav-1 KO NPCs Has No Significant Effect on ERK-1/2 Activity

Fig 44 is a densitometric scan of multiple blots. The bars represent the ratio of pERK/tERK following 1hr DEX exposure. No significant effects of DEX treatment on pERK-1/2 levels are observed. Error bars are +SEM. (*n*=3).

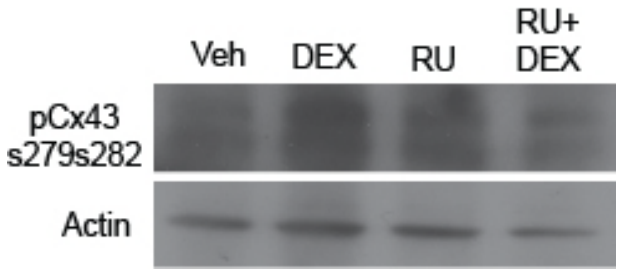

Figure 45: pCx43s270s282 Levels in Cav-1 KO NPCs Following 1hr DEX Exposure

Total protein lysates from Cav-1 KO NPCs treated for 1hr with ethanol vehicle, 100nM DEX, and/or 1µM RU-486 were subjected to Western blot analysis to assess phosphorylated-Cx43 (pCx43s279s282) and Actin. Fig 45 is a representative Western blot. (*n*=3).

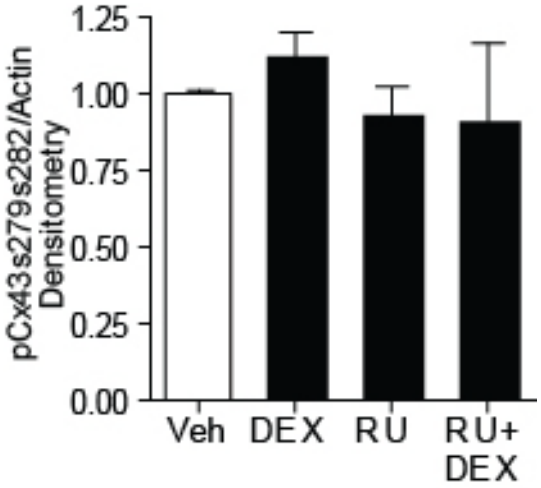

Figure 46: 1hr DEX Exposure of Cav-1 KO NPCs Has No Significant Effect on pCx43s279s282

Fig 46 is a densitometric scan of multiple blots. The bars represent the ratio of pCx43s279s282/Actin following 1hr DEX exposure. No significant effects of DEX treatment on pCx43s279s282 levels are observed. Error bars are +SEM. (*n*=3).

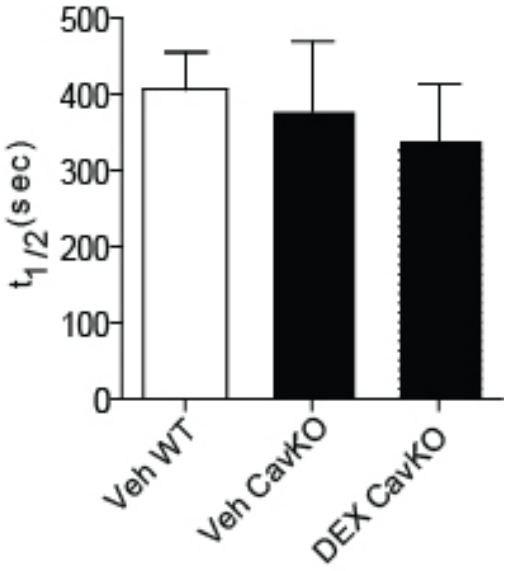

Figure 47: 1hr DEX Exposure of Cav-1 KO NPCs Does Not Alter GJIC

Cav-1 KO and WT NPCs preloaded with Calcein AM were subjected to the following treatments: Ethanol vehicle (Veh) or 100nM DEX (DEX). Mean values for $t_{1/2}$ +SEM of recovery were obtained by fitting a decayed exponential to individual fluorescence recovery curves. No effects of DEX exposure were observed on GJIC in Cav-1 KO NPCs (*n*=4).

4.0 DISCUSSION

4.1 SUMMARY OF RESULTS

In this report, we identify a non-classical and non-genomic GR signaling pathway that impacts NPC proliferation *in-vitro* through inhibitory effects on GJIC. Phosphorylation of specific connexin proteins has been shown to regulate GJIC in other systems. In agreement, we provide evidence for rapid activation of ERK-1/2 by GCs that triggers site specific phosphorylation of Cx43, a major component of NPC gap junctions. This phosphorylation event, in turn, leads to reduced GJIC. Interestingly, GCs do not appear to influence Cx43 (or Cx26) protein expression or subcellular trafficking in murine NPCs. Rapid GR-dependent activation of ERK-1/2 requires a c-src family member and may be initiated by a signaling complex assembled at the plasma membrane through GR interactions in lipid rafts containing caveolin-1. Our studies corroborate the role for caveolin-1 in mediating the anti-proliferative effects of GCs that was established previously in MEFs from caveolin-1 knockout animals (Jasmin et al. 2009). In addition, we identify a novel downstream target of this signaling, GJIC, in a progenitor cell population that could utilize GJIC and/or connexins to maintain synchrony of cell division.

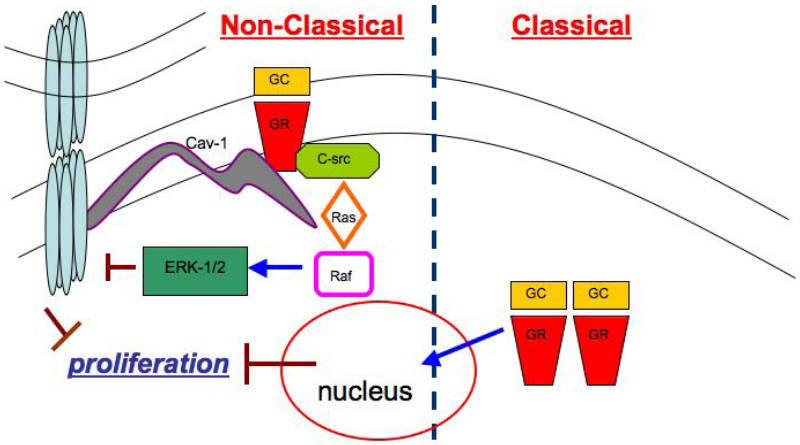

Figure 48: Activation of non-classical and classical pathways by GCs alter NPC proliferation

Hormone (GC) treatment leads to rapid signaling by membrane GR associated with cav-1. Rapid activation of c-src leads to ERK-1/2 activation, phosphorylation of Cx43, and reduction of GJIC. This non-classical signaling reduces s-phase entry. In addition, DEX activates classical, transcription-dependent processes that also reduce NPC proliferation.

4.2 NON-CLASSICAL MEMBRANE GR SIGNALING

Both sucrose gradient fractionation experiments as well as co-IP studies reveal an association between cav-1 and GR and localization of GR to cav-1 enriched portions of the plasma membrane. We also observed that a membrane impermeable DEX-BSA elicits a reduction in GJIC, suggesting that a membrane GC receptor underlies the rapid effects that we have documented. Finally, we demonstrated that ERK-1/2 activation, Cx43s279s282 phosphorylation, and a reduction in GJIC were absent in 1hr DEX exposed Cav-1 KO NPCs. These observations raise a few interesting questions regarding the nature of the GR underlying these effects. First, how does the receptor reach the membrane? Second what is the precise role

of cav-1 in facilitating non-classical GR signaling? Third, is the membrane GR similar to the nuclear GR (GRα)?

4.2.1 Palmitoylation of Membrane Hormone Receptors

Both the presence of GR on the plasma membrane as well as its association with cav-1 bears many similarities to the other major classes of steroid hormone receptors such as ER, PR, and AR. The mechanisms of plasma membrane association and signaling that have previously been identified for these receptors may therefore provide important insights into plasma membrane initiated signaling by GR. For example, a conserved nine amino acid sequence containing a cysteine residue in the ligand binding domain (E-domain) of ERα, ERβ, PR, and AR seems essential for their plasma membrane localization (See Fig 4, Introduction). This particular cysteine residue is subject to palmitoylation, and this form of lipidation seems to be a necessary prerequisite for plasma membrane localization. In addition, mutation of hydrophobic amino acids at +5/6 position relative to the cysteine, phenylalanine or tyrosine residues at the -2 position also significantly reduced membrane localization of these steroid receptors (Pedram et al. 2007). Cav-1 has been hypothesized to be essential for localization of receptor to cav-1 enriched rafts within the plasma membrane and for transport of palmitoylated receptor to the membrane (Levin 2009).

Apart from the presence of the palmitoyl group, the membrane version of these receptors is identical to the nuclear versions (Pedram et al. 2007). For example, transfection of ER null cells that lack both nuclear and membrane ER with ERα or ERβ leads to both membrane and nuclear localization, and membrane ER isolated from breast cancer cells was identical to ERα by

mass spectroscopy (Hammes and Levin 2007). Interestingly, GR also contains a very similar nine amino acid sequence in its E-domain, including a cysteine at the 3 position (Groeneweg et al. 2011). In responses to the questions posed earlier, this suggests the possibility that the membrane GR is also a similarly palmitoylated version of the nuclear GR, and that like AR, PR, and ER, depends on its association with cav-1 to localize and function as a membrane receptor.

4.2.2 MR a Perfect Partner for GR?

Interestingly, one member of the nuclear steroid hormone superfamily that lacks the conserved nine amino acid sequence in its E-domain, including the cysteine residue, is the other GC receptor, the MR (Groeneweg et al. 2011). MR has been shown to be important in rapid non-classical signaling and has a similar motif in its N-terminal domain to the ERα sequence that was shown to be important for ER interaction with cav-1 (Freeman et al. 2005; Groeneweg et al. 2011). Taken together, these findings suggest that rapid MR signaling may be localized to lipid rafts and involve cav-1, but that localization of the MR to the cell membrane utilizes a distinct mechanism than GR. This difference may be an important factor in determining the differential responses of GR and MR to GC in non-classical signaling. For example, palmitoylation of the GR may lead to GR localization in a distinct compartment within cav-1 containing lipid rafts. This may, in turn, influence the signaling cascades activated by GR vs. MR in response to the same ligand (GC).

Furthermore, the potential requirement of palmitoylation for GR but not MR membrane localization necessarily means that a unique subset of cell machinery is required to integrate GR versus MR at the cell membrane. This difference may be highly influenced by the physiological state of the cell (i.e. type of stimulation, extracellular milieu, etc.) necessary for receptor

membrane localization. The timing, the duration, and the speed at which the receptor reaches the membrane could also be affected by this difference. These factors may partly explain reports documenting differences in GC mediated non-classical signaling arising from MR vs. GR (Groeneweg et al. 2011). For example, both GR and MR are expressed in the hippocampus and non-classical MR signaling was found to increase mEPSC frequency. GR knockout did not alter this effect, confirming that this was an MR dependent phenotype. In contrast, non-classical GR action was shown to underlie a corticosterone-dependent increase in hippocampal spine density. In the basolateral amygdala, non-classical MR increased mEPSC frequency following a single administration of GC. A second GC exposure *decreased* mEPSCs, but this effect was mediated through GR, and also occurred through a rapid non-classical mechanism (Groeneweg et al. 2011). As a final point, the diversity of GR vs. MR responses to the identical ligand is not without precedence and is also the case for genomic signaling from these receptors. Despite sharing almost identical DNA binding domains, GR and MR only have 30% overlap in terms of the genes they activate (Groeneweg et al. 2011). It is therefore not entirely surprising that similar principles may also hold for non-classical signaling from these receptors.

4.2.3 The Association of GR and Cav-1 During Plasma Membrane Signaling

We also observed that the membrane GR and cav-1 association was not altered by DEX treatment. This is in contrast to the finding that cav-1 displacement occurs following membrane ER activation in MCF-7 cells or following ligand stimulation of membrane tyrosine kinase growth factor receptors. This displacement appears to be necessary for subsequent membrane signaling (Razandi et al. 2002; Hammes and Levin 2007). While in many instances a decreased association between cav-1 and a membrane signaling receptor is observed during activation of a

signaling cascade, this is not always the case. For example, a stronger interaction between ER and cav-1 was observed in vascular smooth muscle (VSM) cells following E2 stimulation and activation of membrane ER signaling. This was, however, followed by an ER-mediated inhibition of ERK-1/2 signaling (Razandi et al. 2002). We did not detect a change in the observed association of cav-1 and GR by co-IP in response to hormone stimulation. Our results therefore suggest that hormone effects on NPC GJIC and proliferation may be mediated by conformational changes in GR that alter receptor interactions with components of the MAPK pathway, including, for example, c-src family members.

This is in part similar to the result observed following E2 stimulation of membrane ER in VSM cells. However, we documented an increase in ERK-1/2 activation. Interestingly, inhibition of ERK-1/2 in VSM cells following membrane ER activation was associated with a reduction in VSM cell proliferation (Razandi et al. 2002). The GC-mediated increase in ERK-1/2 activity that we observed in NPCs is associated with a similar physiological outcome; namely a decrease in the rate of NPC proliferation.

Cell-type specific differences in targets may therefore underlie the contrasting effects on ERK-1/2 resulting from membrane steroid hormone signaling in VSM cells vs. NPCs. In the case of VSM cells, ERK-1/2 inhibition leads to a reduction in activation of cell cycle target genes such as cyclin D1, thus inhibiting proliferation (Razandi et al. 2002). In NPCs, Cx43 is a downstream target of GC-activated ERK-1/2 with site-specific phosphorylation associated with reduced GJIC, which, in turn, contributes to decreased proliferation.

4.3 INTEGRATION OF CLASSICAL AND NON-CLASSICAL GR SIGNALING

The rapid activation of both non-classical and classical signaling pathways by GR may account for the decrease in NPC proliferation (i.e. S phase progression) and increased cell cycle exit (i.e. as assessed by Ki67 immunoreactivity) brought about by a transient (1hr) DEX exposure. A transient inhibition of GJIC by 1-heptanol in the absence of GR activation also reduced S phase progression of NPCs, but did not affect cell cycle exit. Non-classical and transcription-independent GC effects mediated by a reduction in GJIC may lead to a decrease in the rate of cell cycling in S-phase, whereas transcription-dependent effects, including some that have been previously characterized, may force NPCs to exit the cell cycle entirely (Sundberg et al. 2006). In the following sections, I will explore the possible interaction between non-classical and classical effects on NPC proliferation in greater detail.

4.3.1 Ca^{2+} Waves and NPC Proliferation

In light of our observation of GJIC inhibition, it is interesting to speculate whether the decreased rate of S-phase progression that we have observed in NPCs results from a loss of Ca^{2+} wave propagation. The propagation of spontaneous Ca^{2+} waves through gap junction hemichannels has been proposed to be an essential component of neuronal proliferation in the developing cortex (Weissman et al. 2004). In particular, Weissman *et al.* demonstrated that spontaneous Ca^{2+} waves in radial glial cells in the rat embryonic ventricular zone (VZ) are mediated by gap junction hemi-channels. Inhibition of gap junction communication with cbx diminished these waves, which, in turn, reduced VZ cell proliferation (Weissman et al. 2004). In addition, there

are numerous contexts in which Ca^{2+} has been recognized to regulate cell proliferation (Berridge et al. 2000). The primary role of Ca^{2+} in this setting is in activating Ca^{2+} responsive transcription factors, such as NFAT, that influence cell proliferation. These findings in conjunction with our observations of a loss in GJIC, and a decreased rate of S-phase progression, raise a few interesting possibilities.

For example, GC-mediated reduction of GJIC may inhibit or alter spontaneous Ca^{2+} waves in NPCs akin to the waves that Weissman and colleagues observed in the embryonic rat VZ. The importance of Ca^{2+} release in NPC proliferation was also shown by Lin *et al.*, who demonstrated that Ca^{2+} release was dependent on ATP activation of P2Y1 surface receptors on neurospheres (Lin et al. 2007). Interestingly, ATP was emitted in spontaneous bursts from the proliferating NPCs, and this release was decreased in serum-exposed NPCs that were beginning to differentiate. In addition, loss of the calcium wave, inhibition of the upstream P2Y1 receptor, or inhibition of ATP release were each found to diminish cell proliferation, demonstrating an intimate link between these players and cell cycle progression (Weissman et al. 2004; Lin et al. 2007).

However, Lin *et al.*, 2007 did not address the mechanism whereby ATP exits the NPCs prior to binding P2Y1 receptors. Taken in combination with our results and the observation that Ca^{2+} waves themselves may be dependent on gap junction channels, this suggests a potentially central role for gap junctions in regulation of NPC proliferation. Specifically, gap junction channels and hemi-channels may facilitate the release of ATP into the extracellular space, which binds P2Y1 receptors and leads to the release of intracellular Ca^{2+}. The propagation of the Ca^{2+} wave itself is then dependent on GJIC between adjacent cells. Other small molecules such as IP_3 may also rely on GJIC to travel between adjacent cells. Changes in gap junction communication

therefore may directly affect cell proliferation by altering the passage of multiple metabolites. Importantly, this suggests a critical role for gap junctions in facilitating synchronous activity between proliferating cells.

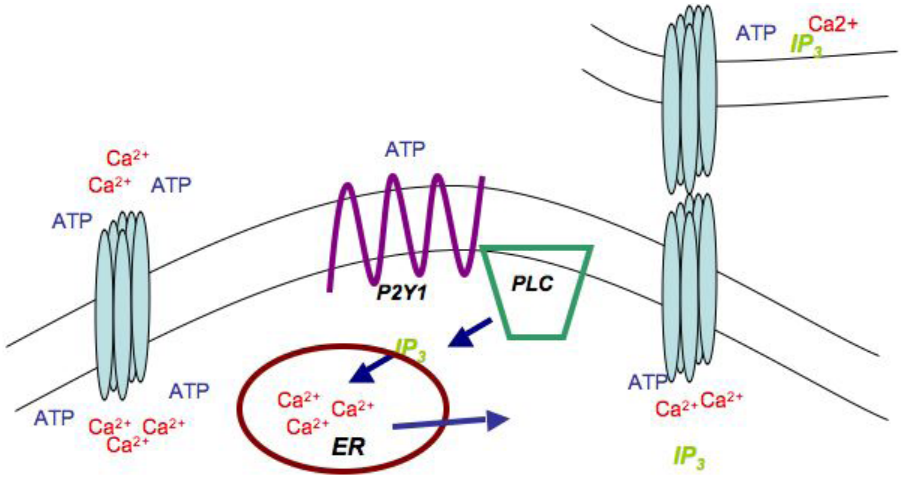

Figure 49: Gap Junctions Facilitate The Movement of Spontaneous ATP and Ca^{+} Waves Needed for Proliferation

Open gap junctions may allow passage of ATP, which binds to and activates P2Y1 receptors leading to IP_3 mediated Ca^{2+} release from the endoplasmic reticulum (ER). The Ca^{2+} itself and the IP_3 could travel between cells in a gap junction-dependent manner and may facilitate cell proliferation.

The observation that Ca^{2+} is an important regulator of proliferation, and the findings by Weissman *et al*., (2004) suggesting that the disruption of Ca^{2+} waves diminishes NPC proliferation, suggests that targets of this ion are particularly important in regulating the cell cycle in neural progenitors (Weissman et al. 2004). In addition, effects on Ca^{2+} may explain the difference in effects on NPC proliferation that we have observed between 1hr GC exposure and 1hr inhibition of GJIC alone with heptanol treatment. In particular, disruption of GJIC alone may interrupt Ca^{2+} induced targets of proliferation and potentially decrease the rate of NPC S-phase entry.

However, GC exposure not only affects Ca^{2+} targets via gap junction effects, but may additionally force the cells out of the cell cycle by activating anti-proliferative GR target genes. The alterations in Ca^{2+} signaling arising from a reduction in GJIC may affect Ca^{2+} dependent transcription factors such as Nuclear Factor of Activated T-cells (NFAT) and cAMP Response Binding Element (CREB) (Berridge et al. 2000).

4.3.2 NFAT- A Potential Target of Disrupted GJIC and Ca^{2+}

NFAT refers to a class of multiprotein complexes that act as transcription factors (Berridge et al. 2000). A number of NFAT isoforms require activation via a Ca^{2+}-calmodulin-calcineurin pathway for nuclear import and trans-activation of target genes (Karpurapu et al. 2009). In the presence of an elevated concentration of Ca^{2+}, the calmodulin-calcineurin complex becomes activated and dephosphorylates NFAT, leading to its nuclear translocation and transcriptional regulation of target genes (Berridge et al. 2000).

Cyclin D1 has been shown to be an NFAT target gene that has particular relevance to cell proliferation. Studies of proliferation in human aortic smooth muscle cells revealed that cyclin D1 has NFAT binding elements in its promoter region. Activation of the upstream calmodulin pathway has no effect on cell proliferation or cyclin D1 expression when NFAT is knocked down (Karpurapu et al. 2009).

The NFAT target gene, cyclin D1, inactivates the retinoblastoma (Rb) protein by phosphorylation and promotes the G1-S transition in the cell cycle (Fu et al. 2004). Consequently, over-expression of cyclin D1 is associated with a number of human cancers including subsets of colon cancer, breast cancer, melanoma, and prostate cancer (Fu et al. 2004). It is therefore conceivable that a reduction in GJIC, such as we have observed in NPCs, could

interrupt Ca^{2+} signaling and reduce the rate of S-phase entry by reducing the levels of NFAT transcribed cyclin D1.

In addition, a reduction in cyclin D1 (although purportedly by a ubiquitin-mediated pathway) has been shown to play a role in GC-mediated reductions in NPC proliferation, thus specifically demonstrating the importance of cyclin D1 in NPC proliferation (Sundberg et al. 2006). Furthermore, GJIC has been posited to play a critical role in NFAT-dependent embryonic heart valve development. In NFAT-deficient mice, the outflow valves and the septal structures of the heart fail to form. A remarkably similar phenotype is observed in Cx45 KO animals, and cell-imaging studies revealed that NFAT is restricted to the cytoplasm in the Cx45 KO animals. Even though a direct link between Cx45-mediated GJIC and NFAT activity or the downstream targets of NFAT was not established, the observed effects indicated that the proper development and proliferation of these heart valve cells may depend on Cx45 GJIC dependent NFAT activation (Crabtree and Olson 2002).

It is also known that NFAT is expressed in the developing brain, and disruption of NFAT signaling leads to deficits in neuronal development (Nguyen and Di Giovanni 2008). One example involves the Down's syndrome critical region 1 (DSCR1) protein. DSCR1 is over-expressed in fetuses with Down's syndrome, and leads to inhibition of calcineurin and a reduction of NFAT activity, which has been purported to account for some of the neurodevelopmental abnormalities Down's (Ooi and Wood 2008). Taken together, these findings suggest the possibility that a GC induced loss in GJIC may lead to decreased S-phase progression in NPCs via a Ca+ - NFAT- cyclin D1 dependent process.

4.3.3 CREB- A Second Potential Target of Disrupted GJIC and Ca^{2+}

CREB is another major target of Ca^{2+} that has been shown to have important roles in regulating cell proliferation (Berridge et al. 2000). CREB is a nuclear localized transcription factor that is activated following phosphorylation at ser133 by a number of kinases including the Ca^{2+} activated Ca^{2+}/calmodulin-dependent protein kinases II and IV (CAMKII and CAMKIV) (Berridge et al. 2000). In order to trans-activate target genes, phosphorylated CREB also needs to bind to its Ca^{2+} activated co-activator, CREB-binding protein (CBP) (Hardingham et al. 1998). The CBP-CREB complex has been shown to bind to a CREB response element on the cyclin D1 promoter (D'Amico et al. 2000; Catalano et al. 2009). In addition, CREB is expressed in the developing brain and is known to be important for the survival and proliferation of NPCs (Dworkin et al. 2007; Dworkin et al. 2009). Ca^{2+}-mediated CREB activation may therefore partly drive NPC proliferation through cyclin D1 expression. The loss or alteration of Ca^{2+} following a reduction in GJIC may reduce the number of cells in S-phase through a CREB-mediated process. The alteration of NPC proliferation via effects on Ca^{2+} target genes such as CREB or NFAT may be reversible if GJIC returns to control levels and Ca^{2+} signaling returns to baseline. In addition, the effects of Ca^{2+} target genes on cell cycle progression of NPCs most likely only represent one pathway of GR regulation of NPC proliferation.

4.4 GR TARGETS IN NPC PROLIFERATION

A reduction in GJIC by heptanol exposure for 1hr leads to a reduction in the number of NPCs in S-phase of the cell cycle. In contrast, a 1hr GC exposure, limited by subsequent RU-

486 treatment, forces NPCs out of the cell cycle entirely. This important difference suggests that GC exposure modulates NPC proliferation through a reduction of GJIC as well as through other means. Therefore, while GC-mediated loss of GJIC may affect NPC proliferation via Ca^{2+} effects as hypothesized above, GCs may also have additional, GR transcriptional target mediated effects on NPC proliferation. One potential set of candidate genes for GR effects on proliferation are the established regulators of the cell cycle. GR may up-regulate the expression of factors that cause cell cycle arrest or repress certain factors that promote cell cycling. In particular, GR has been shown to activate the cyclin-dependent kinase inhibitors (CDIs) p27 and p21 and GR has also been shown to repress the expression of the cyclin-dependent kinases (CDKs) CDK4 and CDK6 (Rogatsky et al. 1997).

4.4.1 CDKs and CDIs

CDKs associate with cyclins at particular points in the cell cycle and CDK-cyclin complexes phosphorylate and activate proteins that promote cell cycle progression. CDIs bind to the CDK-cyclin complexes and inhibit their kinase activity, thus inhibiting cell cycle progression (Rogatsky et al. 1997). DEX-activated GR in the SAOS2 human osteosarcoma cell line upregulates the expression of both p27 and p21 and causes a decrease in proliferation (Rogatsky et al. 1997). The p21 promoter lacks a consensus GRE, but is induced rapidly (mRNA peak within 2hrs of GC hormone treatment) even in the presence of translational inhibition with cycloheximide treatment. The promoter does however contain a number of half-GRE-like sequences. In addition, a GR dimerization mutant (i.e. a GR that is unable to form the homodimers that are typically needed for GR mediated transcription) was able to activate p21 induction (Rogatsky et al. 1999). Taken together, these results indicate that ligand-activated GR

directly up-regulates p21 expression in an atypical manner in SAOS2 cells. Specifically, the rapid induction of p21 mRNA despite the presence of only a half-GRE sequence suggests that GR monomers interact with co-activator proteins to directly up-regulate p21.

Increased expression of p27 took 24hrs to peak at the mRNA level and did not occur in the presence of the GR dimerization mutant. This increased mRNA expression was sensitive to cycloheximide treatment. These results suggest that GR activates p27 transcription indirectly (Rogatsky et al. 1999).

In contrast, DEX-activated GR had no effect on p27 or p21 in the U2OS human osteosarcoma cell line, but did repress CDK4, CDK8, and cyclin D3 activity, which also led to a reduction in cell proliferation (Rogatsky et al. 1997). The precise mechanism whereby GR repressed CDK and cyclin activity was not established. However, the deletion of the N-terminal transcriptional activation domain had no effect on ligand-induced GR repression, but deletion of the GR zinc finger domain that is critical for certain GR-nonreceptor protein interactions did abrogate the repression activity. Thus, an interaction between ligand bound GR and an unidentified transcriptional repressor(s) was likely responsible for the effects observed in U2OS cells (Rogatsky et al. 1997). These examples suggest that GR reduces cell proliferation by multiple mechanisms and in a cell type specific manner. GC-mediated inhibition of NPC proliferation may also partly be a result of activation of CDIs or repression of CDKs. Importantly, our data suggest that even a 1hr exposure to DEX is sufficient to induce the transcription of GR target genes (see *GILZ* in results) making GR transcriptional effects on proliferation a plausible outcome even after a GC exposure limited to 1hr.

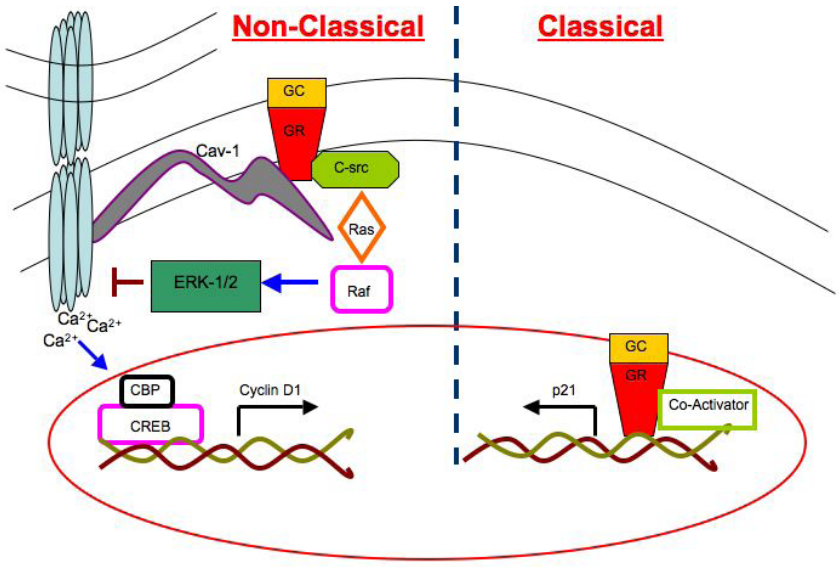

Figure 50: GR May Alter NPC Proliferation by Non-Classical and Classical Mechanisms

Rapid, non-classical GR signaling inhibits GJIC and may disrupt Ca^{2+} waves. This may alter the activity of Ca^{2+}-dependent transcription factors such as CREB that act on cell cycle proteins such as cyclin D1. These effects may be reversible as GJIC returns to pretreatment levels over time. A 1hr DEX exposure may also activate classical GR signaling that up-regulates CDIs such as p21. The combination of classical and non-classical signaling may have a more lasting impact on cell proliferation than inhibition of GJIC alone.

4.4.2 GILZ, a Direct GR Target

GR is also known to directly activate non-cell cycle genes that inhibit cell proliferation. The glucocorticoid induced leucine zipper (GILZ) is a GR target gene with anti-proliferative effects. GILZ is a 137 amino acid leucine zipper (LZ) protein that is induced by GCs and has been most extensively studied in the context of GC effects on immune cells such as T-lymphocytes (Ayroldi and Riccardi 2009). GILZ does not contain a canonical DNA binding domain and has been shown to have cellular effects through interactions with other proteins via its LZ motif (Ayroldi and Riccardi 2009). The GC-induced anti-proliferative effects of GILZ are mediated by binding of GILZ with activated Ras through the Ras tuberous sclerosis complex

(TSC). The Ras-GILZ complex then forms a ternary complex with Raf leading to inhibition of both ERK-1/2 and AKT activity thereby causing a subsequent reduction in cell proliferation (Ayroldi et al. 2007). Silencing of the GILZ gene inhibited the anti-proliferative effect of DEX on T cells. Moreover, GILZ expression inhibited Ras/Raf dependent proliferation and Ras dependent transformation of NIH-3T3 cells (Ayroldi et al. 2007). Interestingly, we observed a rapid increase in ERK-1/2 activity following GC exposure, but this increase is superimposed on a relatively high baseline level. It is possible that while rapid non-classical GR activation decreases S-phase entry via ERK-1/2 mediated effects on GJIC, in the longer term, a GC-GILZ mediated reduction in ERK-1/2 activity may further decrease proliferation through genomic effects. In addition, GILZ inhibition on AKT may further reduce proliferation. This signaling pathway, which we did not analyze in detail in our studies, warrants further attention in future experiments.

4.5 GC EFFECTS ON BIOLOGICAL RHYTMS

A number of recent findings suggest that GC exposure can affect the periodic (circadian and/or ultradian) expression of certain GR target genes (Segall et al. 2009; So et al. 2009). These studies add to a growing body of literature suggesting that biological rhythms are an important aspect of cell physiology, and that disruption of these rhythms can have effects on cell function. Interestingly, a select subset of these circadian genes (e.g. *per1, Npas2, and per2*) is also implicated in cell proliferation in various cell types including NPCs (Borgs et al. 2009; Lee et al. 2010).

4.5.1 Per2 and Proliferation

Per2, which is a transcription factor itself, is a particularly interesting example. Per2 protein has been shown to modulate cell proliferation by directly up-regulating expression of the clock gene *Bmal1* which is also a transcription factor that acts as a negative regulator of the cell cycle related gene *c-myc*. C-myc protein up-regulates cyclin D1; therefore, per2 expression indirectly decreases cyclin D expression and inhibits proliferation by interrupting the G1 to S transition (Borgs et al. 2009). In addition, the per2 promoter was shown to have a GRE. Chromatin immunoprecipitation (ChIP) experiments revealed direct binding of DEX activated GR to the per2 promoter (So et al. 2009). Intriguingly, while DEX exposure caused cycling of per2 mRNA, time course ChIP experiments revealed that GR remained bound to the per2 promoter throughout the period of cycling (So et al. 2009). This suggests that the cycling of per2 results from an event downstream of GR occupancy of the per2 promoter. As a final twist linking per2 with GC-induced rhythms, *per2* gene expression is up-regulated by increases in IP_3 mediated spikes in intracellular Ca^{2+} concentration, suggesting that *per2* expression also may be influenced by alterations in GJIC (Takashima et al. 2006).

Thus, GC exposure may be modulating circadian or rhythmic activity at multiple levels. On the one hand, GC exposure may be influencing the cycling of per2 through genomic mechanisms. On the other hand, GC-mediated inhibition of GJIC may be disrupting Ca^{2+}waves. This may have its own effects on proliferation including the potential modulation of per2 expression.

Interestingly, this kind of multi-level regulation (or dysregulation) of biological rhythms by GCs may partly explain some of the seemingly contradictory results that have been observed when examining the effects of hormone on the periodic cycling of a single factor. In the case of

per2, DEX treatment leads to cycling of mRNA levels over 48hrs; yet examination of GR occupancy of the per2 promoter over the identical time course reveals no significant differences (So et al. 2009). Perhaps this seemingly incongruous result can be explained by GC effects on the cycling of another factor that acts downstream of GR binding to the per2 promoter. In this example, GC-induced changes in Ca^{2+} spikes may interact with per2 at the level of transcription to alter total per2 mRNA levels. In theory, this may result from a Ca^{2+} regulated co-activator or co-repressor of per2 transcription. Work by Takashima and colleagues that identified Ca^{2+} dependent transcription of per2 did not identify the specific transcriptional elements linking Ca^{2+} spikes with per2 transcription. However, their work demonstrated that Ca^{2+} spikes up-regulated per2 expression, suggesting that a Ca^{2+} regulated co-activator or perhaps some transcriptional elements that release repression would underlie this process (Takashima et al. 2006).

4.6 CONNEXINS AND NEURODEVELOPMENT

If the loss of Cx43 mediated GJIC is critical for NPC proliferation, as our studies suggest, then a complete loss of Cx43, such as in a Cx43KO mouse, should have profound effects on the brains of these animals. However, this is not the case. Cx43KO is post-natal lethal due to defects in the ventricular outflow tract and stenosis of the pulmonary artery, but an examination of the post-natal brain of Cx43KO mice reveals no gross abnormalities relative to their wildtype littermates (Dermietzel et al. 2000). Cx43cKO mice that have cre driven by a GFAP promoter also display no evidence of neurodegeneration or astroglial abnormalities at birth. However, mild impairments in motor abilities and an increased predilection for exploratory behavior were reported in adult mice (Frisch et al. 2003; Theis et al. 2003).

4.6.1 Connexins and Compensation

The explanation for this seeming contradiction between our results and the observations noted above may lie primarily in the ability of other Cx isoforms to compensate for Cx43 deficits in the developing brain. For example, Cx43 is the predominant (i.e. most abundant) Cx isoform in astrocytes, but an examination of astrocytes from Cx43KO animals revealed expression of Cx40 and Cx45 by Northern blot, RT-PCR, and immunostaining, although these connexins were also detected in WT astrocytes (Dermietzel et al. 2000). Perhaps more importantly, Cx30 protein expression by Western blot actually increased in Cx43KO astrocytes, indicating the activation of some sort of compensatory mechanism (Theis et al. 2003). Up-regulation of the expression of select connexins, such as Cx30 in Cx43KO astrocytes, together with the sum of baseline function of other minor connexin isoforms may therefore compensate for the loss of a single connexin isoform, even if it is the predominant connexin in that particular cell type. In fact, an examination of GJIC in cells with one of the Cx43 mutations that results in ODDD (discussed in Introduction), revealed that the mutant Cx43 isoform acts in a dominant negative fashion and actually inhibits GJIC from WT connexins (Flenniken et al. 2005). This result indicates that the missense mutation in Cx43, if it compromised Cx43 function alone, may not be sufficient to cause the entire phenotype of ODDD. Rather, the combination of non-functional Cx43 and the inhibitory effect of the mutated Cx43 on the function of other connexins conspires to lead to the complete phenotype of ODDD.

Critically, in instances where compensation for Cx43 occurs, it likely does so over a relatively long time course; at least long enough for the translation of new connexin protein. In contrast, the inhibition of Cx43 mediated GJIC by GC exposure occurs relatively rapidly, with activation of ERK-1/2 seen within two minutes of treatment, and decreased GJIC measured at

1hr. Mechanisms necessary for the compensatory up-regulation of GJIC, such as translation of other connexin isoforms, probably cannot occur in this time window. Moreover, it may be the case that the cellular signaling events that occur when Cx43 is knocked out or when it is mutated so it is non-functional, are distinct from those that occur when Cx43 GJIC is inhibited by phosphorylation. Consequently, the signaling mechanisms that underlie compensation may not be activated during the GC induced reduction of GJIC. In addition, compensatory mechanisms may only occur in certain cell types and in certain cellular contexts. For example, Cx43KO leads to lethal cardiac defects indicating that compensation does not occur in these cells (Dermietzel et al. 2000). In further support of this view, a reduction in Cx43 mediated GJIC in *in-vitro* NPCs resulting from a withdrawal of bFGF persisted even at 24hrs after bFGF withdrawal, the final time point tested (Cheng et al. 2004). This suggests that perhaps neural progenitor cells are particularly susceptible to a reduction in Cx43 mediated GJIC.

Importantly, our results and the findings of others cited above collectively suggest the importance of functional Cx43-mediated GJIC in neurodevelopment. On the one hand, the instances where a loss of Cx43/GJIC leads to compensation by other connexins indicates that functional connexin-mediated GJIC is a biologically important phenomenon. It is important enough from an evolutionary perspective that biologically expensive redundancies (i.e., expression of minor connexin isoforms and the ability to up-regulate other connexins) would be maintained in the event that the major connexin isoform becomes dysfunctional. On the other hand, in instances where compensation for a reduction of GJIC does not occur, as we have observed, there is a pronounced physiological outcome. In our model we observed an alteration in cell proliferation.

4.7 CLINICAL IMPLICATIONS

GC hormones such as DEX are used clinically in a number of contexts. For example, GCs are used in both children and adults for the treatment of autoimmune and inflammatory disorders such as asthma, rheumatoid arthritis, and various allergies (Rhen and Cidlowski 2005). The impact of GCs on development is also exploited for the treatment of complications arising from premature birth and for the treatment of CAH (reviewed in Introduction) (Yeh et al. 2004; Vos and Bruinse 2010). However, the use of these hormones in neonates and antenatally is controversial because of increasing evidence of delayed effects on neurodevelopment (Yeh et al. 2004). For example, infants given DEX for 28 days for respiratory distress syndrome were found to have deficits in motor skills, motor coordination, and IQ at school age (Yeh et al. 2004). In many of these cases, classical GR transcriptional activity is cited as underlying neurodevelopmental effects of GCs. However, our findings support the notion that GC hormones may alter NPC proliferation even following limited exposure to GC by activating a MAPK dependent non-classical signaling mechanism (Ross et al. 2002; Sabolek et al. 2006; Sundberg et al. 2006).

Interestingly, our findings of rapid non-classical and non-genomic effects of GCs on NPC proliferation adheres to an important general principle that has been made in other contexts where rapid non-classical signaling by GR has been observed. In particular, it appears that NPCs utilize non-classical signaling to rapidly initiate a program to reduce cell proliferation, which over a longer time course occurs by classical/genomic mechanisms. This is akin to non-classical GC signaling events in the basolateral amygdala and hypothalamus (reviewed in Introduction- “Non-Classical GR”) where rapid effects occurring in a non-classical and non-genomic manner

appear to be a precursor to similar effects over a longer time course that are mediated by classical GR signaling.

The most straightforward clinical implication of these findings is that exposing the fetal brain to GC hormones even for a short period can activate signaling cascades that may be sufficient to have negative neurodevelopmental consequences. According to this interpretation, the clinical use of GCs should be guided by the need to balance the potential benefits of hormone on lung and heart development versus the certain negative consequences on neurodevelopment. However, this data may also suggest an alternative clinical interpretation that is to some extent supported by the available clinical data. Perhaps there is a time window during which the effects of GC exposure are largely or almost entirely beneficial, but that longer term or more prolonged exposure tips this balance in favor of negative effects of GC exposure. In light of the data in our studies, this interpretation suggests that perhaps the rapid non-classical effects of GC exposure are reversible and temporary, and that the more deleterious consequences occur when classical/genomic programs are activated by more prolonged exposure to hormone. The examination of proliferation from our own studies lends some credence to this idea since inhibition of GJIC alone reduced the number of NPCs actively in S-phase of the cell cycle but did not actually force NPCs to exit the cell cycle entirely. Presumably, cell cycle exit is largely a classical GR effect, whereas decreased S-phase entry is a non-classical effect and may be reversible as long as classical GR signaling events are not activated.

An examination of the clinical literature also suggests that long-term exposure to hormone may at least partly underlie some of the more serious negative consequences of pre and postnatal GC exposure. For example, while DEX has historically been the major GC administered to preterm infants, a growing body of evidence suggests that hydrocortisone may be

a superior clinical alternative due to fewer side effects, including neurodevelopmental side effects (Lodygensky et al. 2005; Benders et al. 2009). Although both DEX and hydrocortisone can cross the blood brain barrier, one of the critical differences between these two hormones is that hydrocortisone can be inactivated by 11β-HSD2, whereas DEX cannot. This enzyme is highly expressed in the placenta as well as in the brain for the majority of gestation (Seckl 2004). Consequently, hydrocortisone administration may primarily activate non-classical signaling mechanisms in the brain before it is inactivated by 11β-HSD2. In contrast, continuous DEX treatment will almost certainly activate classical GR signaling pathways since DEX cannot be inactivated by 11β-HSD2.

The benefits of shorter hormone exposure have also been demonstrated by the treatment of premature infants with pulsatile DEX therapy, instead of continuous DEX. This was shown to be clinically effective in decreasing chronic lung disease and the need for oxygen supplementation and was associated with a reduction in side effects. In this study, infants were given two divided doses of DEX per day for three days, instead of a continuous treatment. While neurodevelopment was not monitored, other common side effects from continuous DEX, such as a significant decrease in weight gain and significant increases in mean arterial blood pressure were not observed (Brozanski et al. 1995). While even a pulse of DEX may activate classical GR signaling mechanisms, pulse therapy presumably leads to a lower fetal DEX concentration between pulses than a continuous infusion, and may therefore primarily activate non-classical GR signaling pathways. These findings are therefore important in two regards: First, they indicate that pulsed DEX dosing can be clinically effective. Second, even though neurodevelopment was not assessed, they indicate that certain side effects are lower from a pulse

treatment. These results warrant a more thorough examination of potential side effects, including on neurodevelopment, from pulse therapy.

In summary, the clinical literature on prenatal and postnatal GC therapy suggests that while hormone treatment has an important, and in many contexts indispensable, therapeutic role, it is also associated with negative side effects. Importantly, the negative effects vary depending on the particular GC used. For example side effects from DEX tend to be more severe than with the use of the natural hormone, hydrocortisone. Combining our results with other findings in the field, we can speculate that the negative outcome is partly a result of the activation of classical GR signaling which may irreversibly inhibit proliferation and/or alter the differentiation of developing NPCs. Therefore, the selective activation of non-classical signaling pathways may preserve the clinical benefits of pre and postnatal GC therapy while avoiding some of the negative side effects. This may be achieved by using natural GCs, pulse therapy of synthetic GCs, or membrane impermeable versions of synthetic or natural GCs that selectively activate non-classical signals.

4.8 CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this thesis identifies a rapid and non-classical GR-mediated inhibition of GJIC in neural progenitors that leads to subsequent alterations in cell proliferation. Importantly, these studies have identified a novel target for membrane-initiated GR signaling in Cx43, and have demonstrated that even a short pulse of hormone can alter cell physiology in the form of proliferation via effects on Cx43-mediated GJIC. Equally importantly, these studies build on and complement previous findings in the steroid hormone and connexin literature. The rapid

signaling cascade appears to be initiated in a c-src dependent manner by a membrane GR that is associated with cav-1. Hormone exposure led to downstream MAPK signaling and eventual ERK-1/2 phosphorylation of Cx43. The signaling cascade that I identified is remarkably similar to non-classical ER signaling that utilizes some of the same protein co-factors (i.e., c-src and cav-1) and results in ERK-1/2 activation. In addition, Cx43 phosphorylation by ERK-1/2 at the consensus ERK-1/2 target sites was known to lead to a loss of GJIC, in agreement with these findings. In a broader sense, my finding also adds to the view that non-classical and classical steroid hormone signaling are intimately linked and lie on a continuum in which activation of one program complements the activation of the other (Haller et al. 2008). In this particular case, rapid non-classical GR activation reduced S-phase progression of NPCs whereas slower acting classical GR activation was most likely responsible for forcing NPCs to exit the cell cycle entirely.

There are also a number of avenues by which the work presented here can be expanded and built upon to further our understanding of GR signaling and its effects on cell physiology. We present evidence suggesting that GR is membrane localized and that it is associated with cav-1. It would be interesting to see if the same mechanisms that underlie membrane localization of AR, PR, and ER also underlie GR membrane localization. In order to address this possibility, the putative "membrane localization" sequence in the E-domain of GR could be mutated to prevent palmitoylation, as done previously by Pedram *et al.*, (2007). We could then assess palmitoylation in the native and mutated GR and also determine if membrane localization occurs in the mutant.

Further use of cav-1 KO cells may definitively demonstrate the role of this protein in GR mediated modulation of NPC proliferation. Studies of cav-1 KO animals have demonstrated an

increase in neural progenitor proliferation in adult mice (Jasmin et al. 2009). Our studies suggest that cav-1 may have a similar anti-proliferative effect in NPCs. It would therefore be informative to examine the rate of proliferation of cav-1 KO NPCs in the presence and absence of hormone. If cav-1 is essential for GR non-classical signaling, we would expect that hormone treatment may not inhibit proliferation or at least S-phase entry following a transient exposure to GC. It would be interesting to document if the level of proliferation in untreated cav-KO NPCs is also elevated compared to WT and to assess if longer (i.e. classical GR) hormone exposure has differential effects on cavKO NPC proliferation. The latter may provide further insight into the role of cav-1 in mediating the divergence of non-classical and classical GR signaling.

The precise structural make-up of the membrane GR signaling complex is not known, and gaining a more complete understanding of what this complex looks like would be very insightful. In fact, even an examination of the ER literature does not clarify exactly how the hormone receptor and proteins such as cav-1 that it is purported to interact with associate within the physical space of the cell membrane. In particular, is "membrane GR" located largely in the cytoplasm by the inner leaf of the cell membrane, within the cell membrane, outside the membrane facing the extracellular space, or some combination of the above? Activation of GR by membrane-impermeable BSA conjugates has been used as evidence that some portion of the receptor faces the extracellular space, but BSA can be internalized within caveolae, thus discrediting this as definitive proof of GR localization outside the cell membrane (Levin 2005).

Immunostaining of non-permeabilized cells has also been used as evidence that the hormone receptor is at least partly extracellular. However, the limited resolution of standard light microscopy, as well the possibility of antibody penetration into caveolae make this a dubious assertion (Levin 2005). More definitive evidence of GR localization at the membrane

may be gained by a combination of high-resolution EM studies as well as computational modeling of GR based on the list of known GR binding partners. The latter technique could provide insight into potential conformational changes in GR within and around the membrane space. Given the clear similarities between the superfamily of nuclear receptors, this sort of study will be particularly useful because of the potential generalizability of the findings.

We also speculate that GR-induced reductions in GJIC contribute to alterations in NPC proliferation through a combination of Ca^{2+}-dependent effects and Ca^{2+}-independent effects mediated by classical GR signaling. An important extension of this study would be to measure the effects of hormone treatment on intercellular Ca^{2+} levels using live cell imaging coupled with a Ca^{2+} sensitive ratiometric indicator like Fura-2. The general methodology of hormone treatment and/or pharmacological inhibition of gap junctions followed by live cell imaging can be extended to examine the role of GJIC and the passage of any given cellular metabolite. For example, measuring ATP release by neurospheres using bioluminescent live cell imaging following blockade of gap junctions with cbx could reveal the importance of gap junction communication in the activity of this metabolite (Lin et al. 2007).

Another important extension of our findings would be to identify gene targets of 1hr GC exposure that alter proliferation. In the discussion section, a few potential candidates were identified based on the existing literature. These targets can be broadly categorized into those proteins that could be altered by hormone effects on GJIC (i.e. Ca^{2+} target genes such as NFAT) and those proteins that potentiate cell cycle arrest by GR action that is independent of GR effects on GJIC. While the approach of identifying a few select targets from the literature and measuring changes in their protein and/or mRNA expression may yield informative results, a less biased approach utilizing gene-chip technology may be especially useful in this setting. For

example, a mini-array focused on cell cycle related and Ca^{2+} modified genes that utilizes cDNA from control, 1hr 1-heptanol, and 1hr DEX treated NPCs could be particularly insightful. The more promising candidates from this type of broad search can then be validated and further probed using more traditional techniques.

There are also a few very specific experiments that could reinforce the results that have been presented in this thesis. Utilization of NPCs from GR knockout animals or knockdown of GR would allow confirmation of the role of GR in the non-classical signaling cascade that has been identified. In addition, while we have shown that ERK-1/2 phosphorylates Cx43 at S279/S282, definitive proof of the role of this site in mediating GR induced reductions in GJIC would necessitate the use of a Cx43 phosphorylation mutant at these sites. Activation of MAPK in cells transfected with this Cx43 mutant should not result in a reduction in GJIC. Finally, while we have established a hormone-dependent reduction in GJIC in NPCs that is present at 1hr, it would be useful to also know more about the dynamics of this process between the moments after hormone is first introduced up to 24hrs later. In this regard, performing FRAP at time points prior to 1hr, such as at 30min, and between 1hr and 24hrs would be insightful. The complementary experiment to this would be measuring Cx43 phosphorylation at s279/s282 at these time points as well.

In a broader sense the objective of these studies was not only to understand the molecular mechanism whereby GCs alter GJIC in NPCs, but to also expand on our understanding of how GCs impact on human health and disease. To this end, these studies have demonstrated that even a transient exposure to DEX leads to a loss in cell proliferation, and that a reduction in GJIC alone, one aspect of hormone exposure, reduces S-phase entry of NPCs. These *in-vitro* results are admittedly far-removed from human clinical practice, but they can inform a program of

experiments that can be clinically useful. An important next step would be to determine the impact of non-classical GR activity *in-vivo* on proliferation in the embryonic murine brain. For example, it would be interesting to administer DEX-BSA to pregnant mice and then determine cell proliferation in the embryonic brain 24hrs later. (Unfortunately, RU-486 cannot be used to limit hormone exposure after 1hr *in-vivo*, as was the case with our *in-vitro* experiments, because it will cause spontaneous abortions.)

While we limited our studies to the use of DEX, the commonly clinically used synthetic GC betamethasone as well as the natural GC, corticosterone, could also be used in both *in-vitro* and *in-vivo* studies. This kind of research program will allow for a determination of differential effects of various GCs and may also show us if the effects we have observed *in-vitro* are reproducible *in-vivo*. In addition, continued *in-vitro* studies as highlighted previously will aid in the dissection of the mechanisms operating in classical and non-classical actions of steroid receptors and complement *in-vivo* studies. This could reinvigorate the search for novel ligands that preferentially activate one pathway, either classical or non-classical, and otherwise provide potential targets that allow for more selective actions of hormone exposure. Given the important clinical role of hormone therapies and the many negative side effects associated with their use, this presents the possibility of selectively eliciting only the positive effects of GC (or other hormone) therapy- an outcome with great therapeutic promise.

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