DISSECTING THE ROLE OF THE GLUCOCORTICOID RECEPTOR IN EMBRYONIC CORTICAL DEVELOPMENT: INSIGHTS FROM *IN UTERO* GLUCOCORTICOID EXPOSURE

by

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Synthetic glucocorticoids (GCs) such as dexamethasone (DEX), are given to pregnant women to reduce the incidence of respiratory distress syndrome and intraventricular hemorrhage in premature infants. However, animal and human studies reveal adverse effects of DEX on cerebral cortical development. Understanding the basis for these negative neurological consequences of prenatal GCs would be aided by a detailed description of the developmental and spatial expression profile of the glucocorticoid receptor (GR) in neural stem/progenitor cells. This could provide mechanistic understanding of the impact of premature activation of GR signaling in the developing brain, particularly since endogenous GC levels are low during the period of fetal development at which DEX is administered. In this study we show that GR protein is expressed in cortical radial glia cells (RGCs) and intermediate progenitor cells (IPCs) from embryonic day 11.5 in the mouse. Furthermore, in many areas of the fetal brain, GR was primarily nuclear at times when endogenous GCs are expected to be low. We also examined the consequences of prenatal DEX exposure and found that a single, clinically relevant DEX dose at midgestation (E14.5) reduced cortical thickness and surface on E17.5, but increased the number of neurons throughout the cortex, including in deep layer VI. BrdU birthdating revealed that the BrdU+ progeny of the E14.5 progenitors was increased by DEX. Furthermore, while a higher proportion migrated in the cortical plate, a smaller proportion remained in the progenitor zone,

suggesting that DEX promoted precocious neurogenesis. Accordingly, after a single, 24 hour exposure on E14.5, DEX induced a neurogenic fate shift in progenitors in an S-phase-specific manner. Specifically in non-S-RGCs DEX promoted the IPC fate, while it induced re-entry of S-phase RGCs and expansion primarily of the neurogenic RGC pool (BrdU+). DEX induced neurogenic divisions of S-phase RGCs and S-phase IPCs causing increase of the BrdU+ cells. FACs analysis verified the neurogenic shift in S-phase BrdU+ progenitors, which accumulated in G0G1 phase. Collectively, these results suggest that premature, DEX-induced activation of GR induces cell cycle phase-dependent precocious neurogenesis and highlights a role for GR in regulating neurogenesis in the developing brain.

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PREFACE

Ithaka

Constantine P. Cavafy

As you set out for Ithaka

hope the voyage is a long one,

full of adventure, full of discovery.

Laistrygonians and Cyclops,

angry Poseidon—don't be afraid of them:

you'll never find things like that on your way

as long as you keep your thoughts raised high,

as long as a rare excitement stirs your spirit and your body.

Laistrygonians and Cyclops,

wild Poseidon—you won't encounter them

unless you bring them along inside your soul,

unless your soul sets them up in front of you.

Hope the voyage is a long one.

May there be many a summer morning when,

with what pleasure, what joy,

you come into harbors seen for the first time;

may you stop at Phoenician trading stations

to buy fine things,

mother of pearl and coral, amber and ebony,

sensual perfume of every kind—

as many sensual perfumes as you can;

and may you visit many Egyptian cities

to gather stores of knowledge from their scholars.

Keep Ithaka always in your mind.

Arriving there is what you are destined for.

But do not hurry the journey at all.

Better if it lasts for years,

so you are old by the time you reach the island,

wealthy with all you have gained on the way,

not expecting Ithaka to make you rich.

Ithaka gave you the marvelous journey.

Without her you would not have set out.

She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.

Wise as you will have become, so full of experience,

you will have understood by then what these Ithakas mean.

C.P. Cavafy, Collected Poems. Translated by Edmund Keeley and Philip Sherrard.

Edited by George Savidis. Revised Edition. Princeton University Press, 1992

As this journey through my doctorate studies ends, I would like to devote a few lines of gratitude to the people that have sparked it, fuelled and supported it through the waves of graduate life, albeit, this is a minute thank you towards the worth of their support.

First, I would like express my gratitude to my advisor Professor Don DeFranco for his support both as a scientist and also as a person, and for guiding me through my Ph.D. journey. Don has been a wonderful mentor, who had faith in me and supported my scientific ventures, providing me with room to explore my ideas however risky they were, while always providing critical advice and input throughout these years. I could not have asked a more supporting mentor and I thank you for that. Also, I would like to express my thank you to my co-advisor, Professor Paula Monaghan-Nichols, who also supported me and provided me with guidance, advice and insights for my research. Paula, is a very energetic person and it has been a great time working with her and experiencing her enthusiasm and love for science.

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Thank you for always nourishing my spirit and always supporting me in my options, and always reminding me to keep my faith to God and to myself. Thank you, for always being next to me, even if we were an Atlantic away. Your unconditional love and support has kept me strong in very difficult times. I love you and I could never ask for better friends and parents. My love and thank you also goes to my brothers Charalampos and Christos, who have always supported me and made me laugh, an invaluable asset, even through hard times. Also, I would also like to express my deepest gratitude and love to my dear Aliki and Eftichios, for their unconditional love and support through the years. Last but not least, my thank you goes to my fiancé, Charalampos Tsourakakis. Without you I could have never completed this journey.

I would like to dedicate this work to two of my loved persons, my dear grandmother, Milia, who did not have the chance to see me grow to a woman, but her loving presence has always been with me, and, to my fiancé, Charalampos Tsourakakis. Thank you for being a beacon in my journey to this Ithaka.

Main abbreviations used in this work: Dexamethasone (DEX), radial glia cells (RGCs), intermediate progenitor cells (IPCs), neural stem/progenitor cells (NSPCs), 5-bromo-2'-deoxyuridine (BrdU), Paired box protein 6 (Pax6), T-box brain protein 2 (Tbr2), ventricular zone (VZ), subventricular zone (SVZ).

INTRODUCTION

1.1 GLUCOCORTICOIDS AND THE GLUCOCORTICOID RECEPTOR

1.1.1 *Glucocorticoids: Historical Perspective*

Glucocorticoids (glykos, Greek: sweet + cortex, GCs), are steroid hormones produced by the zona fasciculata of the adrenal cortex. These hormones are indispensable for life, since they act in nearly all organ systems and cells of the body. They regulate a multitude of processes spanning many organizational levels: from regulation of the cell cycle, organ development including the brain, cardiovascular function, metabolic and immune processes, reproduction and cognitive functions. Due to their powerful immunosuppressive properties, GCs and their synthetic forms (sGCs), are amongst the most prescribed drugs for the treatment of inflammatory and auto-immune diseases such as allergies, rheumatoid arthritis and multiple sclerosis ((Oakley and Cidlowski, 2013) and references therein). Also, they have been used along the first line of defense in cancer and especially in cancers of the lymphoid system such as leukemia and lymphoma. The immunosuppressive effects or cortisone, the precursor molecule of cortisol (or hydrocortisone), were first discovered by Philip Showalter Hench (MD at the University of Pittsburgh and then of Mayo Clinic) when he showed that it can alleviate the pain symptoms associated with rheumatoid arthritis. Along with Tadeus Reichstein and Edward Calvin Kendall,

they shared the Nobel Prize for Physiology or Medicine in 1950, for discovering the production of cortisone in the adrenal glands, its structure and functions (Figure 1). GCs are instrumental in the stress response, first noted by Hans Selye in 1936 when he published a not more than a half-page report in Nature noting the common characteristics of animals exposed to various noxious stimuli, including enlargement of the adrenals [1], later named by Selye as the "stress syndrome". Hans Selye, also discovered the Hypothalamus-Pituitary Adrenal axis (HPA axis), which controls the stress response. In addition, Selye proposed that GCs are critical regulators of homeostasis or of the *internal mileau*, which was first noted by Claude Bernard and then adapted by Walter Cannon. Cannon is credited for the proposal of the "fight or flight response" and the integration of the central nervous system response with the adrenal function to maintain homeostasis[2] (Figure 2).

[1] http://brainimmune.com/hans-selye-and-the-birth-of-the-stress-concept/

[2] http://brainimmune.com/walter-cannon-homeostasis-the-fight-or-flight-response-the-sympathoadrenal-system-and-the-wisdom-of-the-body/

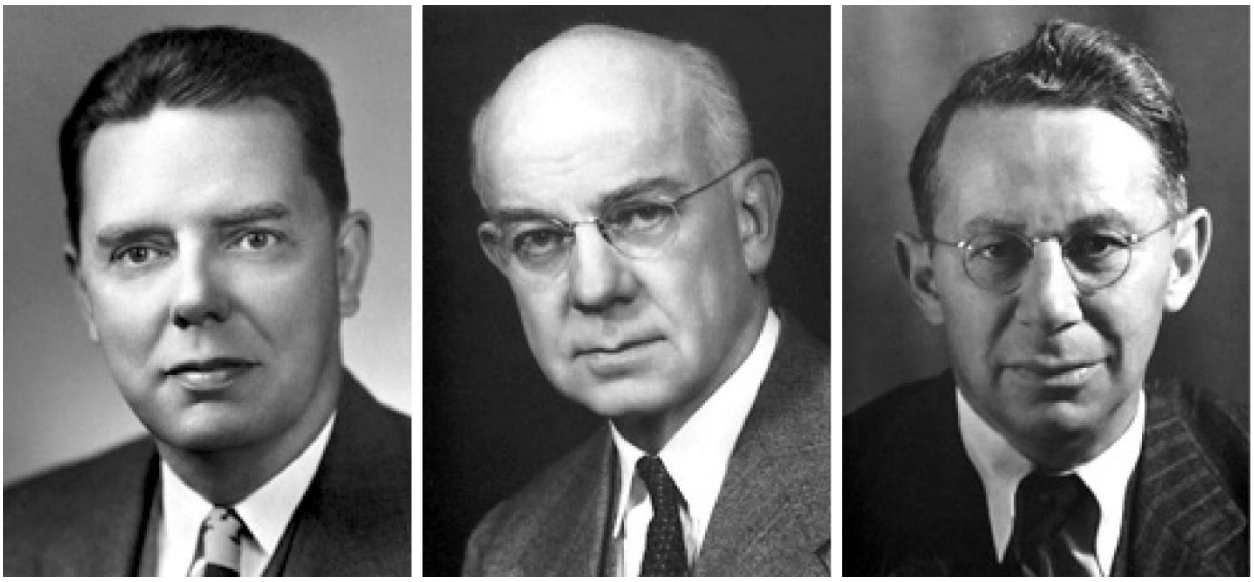


Figure 1. Philip Showalter Hench, Edward Calvin Kendall and Tadeus Reichstein

They shared the 1950 Nobel prize in Medicine "for their discoveries relating to the hormones of the adrenal cortex, their structure and biological effects". Source "The Nobel Prize in Physiology or Medicine 1950". Nobelprize.org. Nobel Media AB 2013.

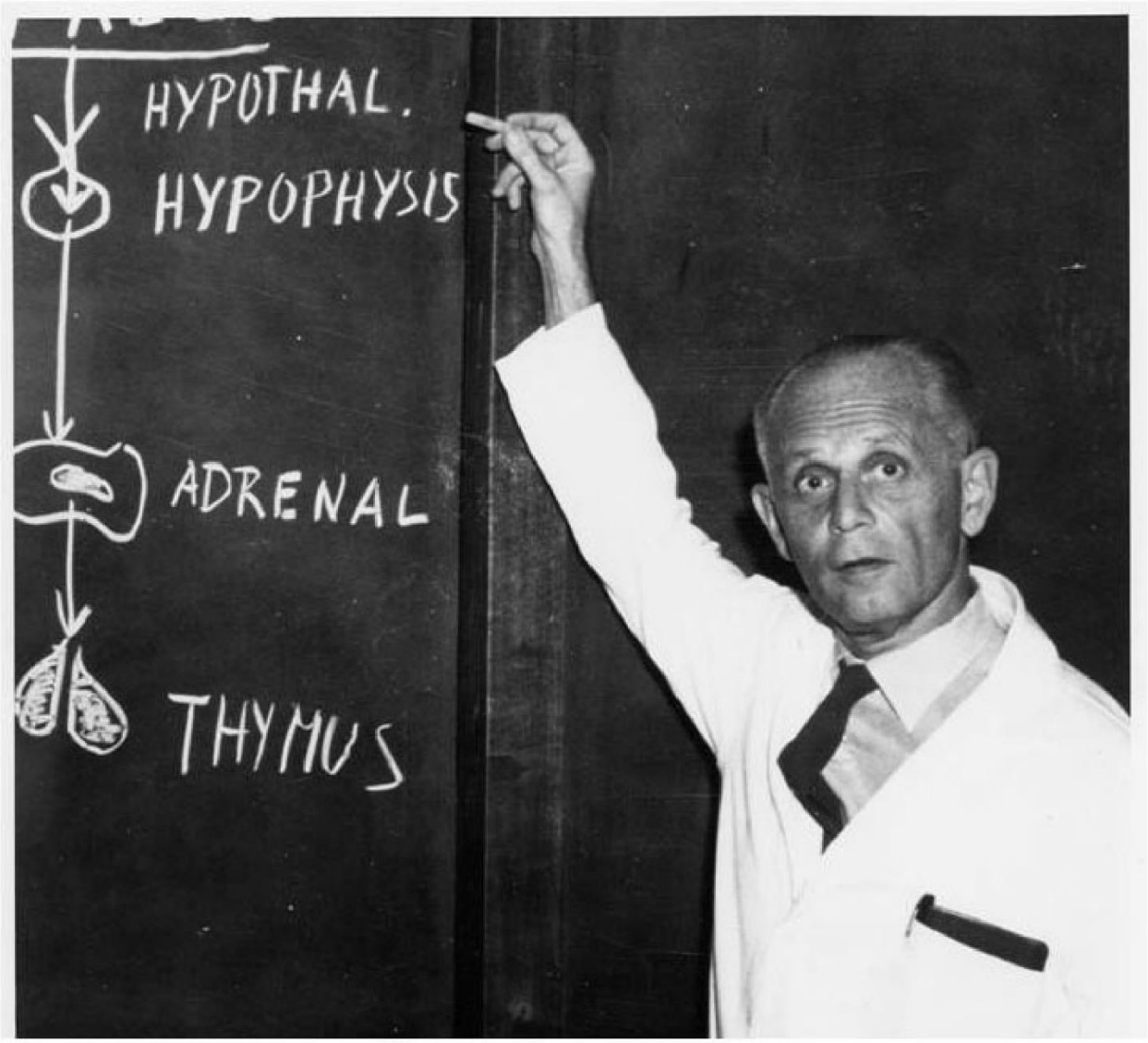


Figure 2. Hans Selye and the Hypothalamus-Pituitary-Adrenal Axis (Adapted from Berczi, 2009)

1.1.1.1 *Steroidogenesis in the embryo*

Cortisone is synthesized *de novo* by cholesterol. The generation of the active form of the hormone, cortisol in primates and corticosterone in rodents, is catalyzed by 11β -hydorxysteroid dehydrogenase type 1 (11β-HSD1) (Figure 3B). This reaction is bidirectional, whereby, cortisol can be degraded to its inactive keto-forms cortisone and 11-dehydrocorticosterone by 11β-HSD2. In the human placenta, 11β-HSD2 is expressed in high levels and protects the embryo from exposure to high levels of maternal GCs (Benediktsson et al., 1997). The human fetal adrenal can produce *de novo* cortisol in the fetal zone, a specialized section of the fetal adrenal, from the first trimester. The production is transient and the physiological significance of cortisol production at this stage is not clear. The expression of 2 3β-hydroxysteroid dehydrogenase/Δ^{4-5} isomerase (HSD3B2), which is required for *de novo* GC production, is suppressed and its levels start to increase in the fetal adrenal approximately from week 24 and is more pronounced in late-gestation (Ishimoto and Jaffe, 2011). This late increase in GC production, reflects the growth stimulating property of GCs, as they promote organ maturation, stimulation of lung surfactant production and prepare the fetus for forthcoming birth. In the mouse, which is used by many groups as a model for studying the GC actions, endogenous production of GCs in the embryo is established around E16 (Venihaki et al., 2000). The morphogenesis and function of the human adrenal is largely driven by ACTH, although these results need still more investigation, since their majority have been generated *ex vivo* or *in vitro*. The placenta (or better ascribed as the feto-placenta unit) plays a key role in fetal adrenal development and the activation of the HPA axis. A hypothetical model of how this happens is suggested by Ishimoto & Jaffe (2011). Close to term the feto-placental unit secretes Corticotropin-releasing hormone (CRH) stimulating cortisol production in the fetal adrenal and upregulation of ACTH receptors (ACTHR) and

thereby sensitizing the fetal adrenals to ACTH and thereby establishing the fetal HPA axis. Cortisol promotes organ maturation including the lungs (Bolt et al., 2001), while placental CRH and cortisol stimulate the increase of prostaglandins which initiate the process of parturition. For a detailed review of the human adrenal development the reader is directed to the review by Ishimoto & Jaffe (2011). In the mouse, the HPA axis feedback loop, is established at E16 (Reichardt and Schutz, 1996), coincidently with the initiation of the production of endogenous GCs. Different internal or external signals such as circadian rhythms or stress induce the release of CRH from the paraventricular nucleus of the hypothalamus, which then is transported through the portal circulation to the anterior pituitary and promotes synthesis and secretion of ACTH. Subsequently, ACTH acts on the adrenal cortex and promotes the production of GCs. Due to a negative feedback loop between the Hypothalamus-Pituitary and Adrenals, increasing levels of GCs inhibit the secretion of CRH in the hypothalamus and ACTH from the pituitary. This negative loop is established by GC action on glucocorticoid receptors (GRs), (Figure 3A).

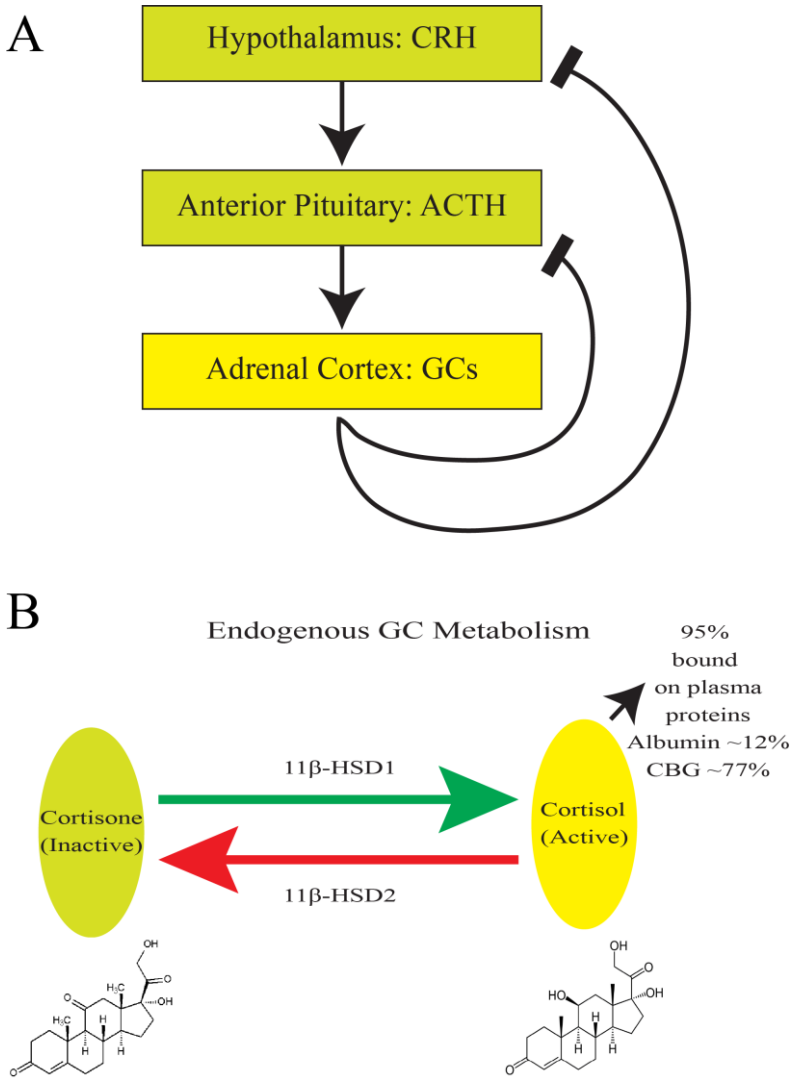


Figure 3. The Hypothalamus Pituitary Adrenal (HPA) Axis and GC metabolism

A. High levels of GCs exert a negative feedback control on the hypothalamus and pituitary and inhibit their stimulatory effect on the adrenals until GC levels return to equilibrium. ***B.*** Cortisol is degraded to inactive cortisone by 11β-HSD2 and vice versa by 11β-HSD1. 11β-HSDs along with plasma proteins, regulate the levels of available bioactive cortisol. Placental 11β-HSD2 is particularly important during the embryo development, since it degrades the maternal GCs and protects the embryo by potential damage from high maternal GCs.

1.1.2 *GR Biology and Signaling*

The intracellular effects of GCs are mediated through the corticosteroid receptors: the GR and the mineralocorticoid receptor (MR). MR has a greater sensitivity to cortisone than GR, and can be activated by lower concentrations of GC but is also responsive to mineralocorticoids such as aldosterone, hence its name. Its expression is more restricted compared to GR which is expressed in almost all cells of the body, therefore GR is the primary mediator of GC actions. GR belongs to the family of steroid nuclear hormone receptors and in the subfamily of nuclear receptors (NRs), hence its gene symbol NR3C1. The human GR is encoded on chromosome 5 between loqi 5q31-q32 (Hollenberg et al., 1985), whereas in the mouse the GR gene is located on chromosome 18 (Danielsen et al., 1986).

The GR protein has a modular structure with 3 main domains: the *N-terminal transactivation domain* (NTD), the central *DNA-binding domain* (DBD) which is separated by the C-terminal ligand-binding domain (LBD) by a flexible hinge region (H), (Figure 4). GCs are bound by a hydrophobic pocket which is formed by twelve alpha-helices and four beta-sheets within the LBD. Nuclear localization of the receptor is controlled by two *nuclear localization signals* NL1, located within the DBD/hinge region and NL2 which is in the LBD. The NL1 signal has been associated with a ligand independent localization of GR, which is dependent on the cell cycle phase (Matthews et al., 2011). The receptor harbors two transactivation domains AF1 located in the NTD and AF2 found in the LBD. The latter is associated with ligand-dependent transactivation of the receptor whereas the former has recently been associated with ligand independent GR signaling (Matthews et al., 2011). Within the DBD, two zinc finger motifs recognize special DNA elements within target genes that are called *glucocorticoid responsive elements* (GREs). The consensus GRE sequence is an imperfect palindrome composed by two

half-sides, 6 bp each: GGAACAnnnTGTTCT. The ligand-activated GR binds these sequences as a homodimer and promotes transcription of target genes, and the spacer region (nnn) is instructive for the GR homodimer to bind the DNA. However, GR can also repress gene transcription and this mechanism is associated either by interactions with other cofactors, changes in the chromatin landscape or by binding to negative-GREs. The sequence of negative-GREs differs from the sequence of the canonical GRE, in that the spacer is of variable size: CTCC(n) $_{0\text{-}2}$ GGAGA. Also, two GR receptors bind the nGRE but do not homomerize (Hudson et al., 2013; Lightman and George, 2014; Oakley and Cidlowski, 2013; Surjit et al.). The chromatin landscape is a critical regulator of GR action, since the majority of GR binding sites are within strongly DNase I–sensitive regions of the genome, in hormone-free conditions (John et al., 2011). This is very important considering the growth-promoting role of GCs, and also because chromatin remodeling is a dynamic process and integral determinant of cell fate determination during development, including brain development (Ho and Crabtree, 2010; Lessard et al., 2007). Moreover, differences in chromatin accessibility may underlie the tissue-specific effects of GCs.

1.1.2.1 *Classic GR signaling*

In the classical scheme of GR signaling, the GC-free receptor is located in the cytoplasm of the cell in association with chaperone proteins and immunophilins such as FKBP51 and FKBP52. Once the receptor, binds to a GC ligand then the protein complex is dissociated and the GC-GR complex is shuttled into the nucleus to act on gene targets, either by inducing or suppressing their expression. Moreover in addition to binding directly to the DNA as a homodimer, GR can regulate gene expression through physical association with other transcription factors and by binding directly on the DNA or indirectly through regulation of the associated co-factor function.

The transcriptional effects of the classical GR signaling pathway require substantial time to take place that can range up to hours. Interestingly, at high concentrations of GR, the receptor can form hormone-free dimers and bind DNA, which may enhance transactivation potency of the receptor following a subsequent GC exposure (Robertson et al., 2013).

1.1.2.2 *Non-Classical Rapid GR signaling*

Beyond the classical GR pathway which is executed by nuclear GR, membrane associated GRs can mediate rapid effects of GCs in great part through kinase cascades that do not primarily require changes in gene expression. Changes in cellular processes as a result of rapid GR signaling can be initiated as rapidly as a few seconds (Lau et al., 2013; Oakley and Cidlowski, 2013{Popoli, 2012 #254; Popoli et al., 2012). Fast actions of GC-GR signaling have been studied within the context of the effects of stress on neurotransmission (Tasker et al., 2006). In the prefrontal cortex, a GR-specific mechanism participates in endocannabinoid production and release regulating in this manner synaptic function of the affected neurons (Popoli et al., 2012). Dexamethasone (DEX), a specific GR agonist, induces an increase in the dendritic spines in CA1 pyramidal neurons within 1 h, in adult male rat hippocampus (Groeneweg et al., 2011; Komatsuzaki et al., 2005; Komatsuzaki et al., 2012). Also, DEX can rapidly induce the cell surface expression of the serotonin transporter in embryonic stem cell-derived serotonergic neurons (Lau et al., 2013). In addition, rapid GR signaling can modulate GABA-ergic neurotransmission in the rat prefrontal cortex and hippocampus and thus affect spontaneous activity in these areas (Teng et al., 2013). Membrane-bound GR in association with caveolin 1 (Cav1), can directly affect cell cycle progression in neural stem cells in culture and in A549 lung epithelial cells (Matthews et al., 2008b; Peffer et al., 2014; Samarasinghe et al., 2011). Activation of GR by DEX, promotes phosphorylation of Cav1 and protein kinase B (PKB)/Akt

via the src kinase and leads to growth arrest and accumulation in G1/S (Matthews et al., 2008b). This pathway is also functional in neural stem cells *in vitro* and regulates their proliferation (Peffer et al., 2014; Samarasinghe et al., 2011). Moreover, in the A549 cells, Cav1-dependent rapid GR signaling, promotes the activation of glycogen synthase kinase-3β (Gsk-3β) and of the mammalian target of rapamycin (mTOR) implicating further the PI3K kinase pathway in the rapid effects of GCs.

1.1.2.3 *Ligand independent GR signaling*

Beyond its classic role as mediating the effects of GCs, the GR is itself a transcription factor. As discussed above, the GR can regulate chromatin structure and in association with other transcription factors or *co-factors*, can modulate gene expression. It was recently shown that GR can function in the absence of GC binding to regulate gene transcription. Specifically, GR directly binds the promoter of the BRCA1 gene in association with the co-factor GABP, and promotes BRCA1 expression in mammary cells. This relation is specific to GC-naïve conditions and is abolished in the presence of hydrocortisone (Ritter et al., 2012). Furthermore, microarray analysis revealed that this basal regulation of gene expression by GR in the absence of GCs is not limited to BRCA1 but applies to many other genes, which are critical for organ development such as cyclin D2 (Ritter and Mueller, 2014). Moreover in proliferating cells, the subcellular localization and function of the unliganded GR is tightly regulated by the cell cycle phase (Matthews et al., 2011). NL1-dependent nuclear accumulation of GR occurs throughout the interphase (middle G1-G2 phases), whereas during mitosis and until early G1 the receptor is excluded from the nucleus. Ligand-independent transactivation of the receptor is induced during G2/M by phosphorylation of GR on the Ser211, which is located within AF1. These data suggest

that the subcellular localization of GR under basal conditions in proliferating cells is tightly linked to GC response.

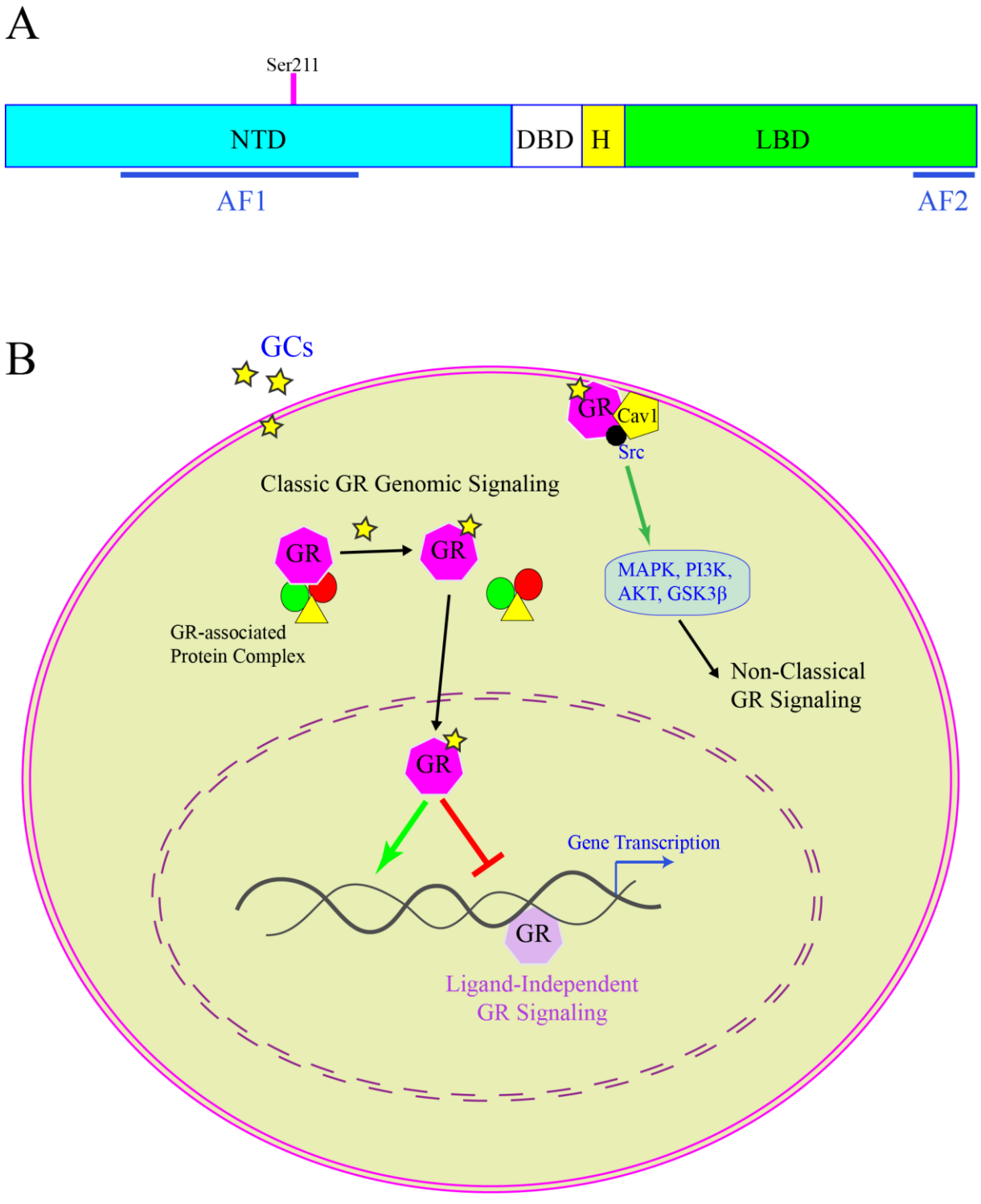


Figure 4. Modular structure of the GR protein and GR signaling, classical and non-classical.

A. The GR protein consists of the N-terminal domain (NTD) which contains the activation domain 1 (AF1), the DNA-binding domain (DBD), a hinge region (H) and the carboxy-terminal Ligand-Binding Domain (LBD). The LBD also contains an AF (AF2). The NTD domain is the target for many post-translational modifications mainly of phosphorylation on Serine residues. Shown is Ser211, which is very important for ligand-independent function of GR. ***B***. The classical and non-classical GR signaling are ligand-dependent (ligand are depicted with stars). The classical or genomic signaling pathway is mediated by cytosolic GR, which after GC-binding, translocates in the nucleus and promotes changes in gene transcription. The non-classical or rapid non-genomic pathway is mediated by membrane bound GR, is executed through kinase cascades and does not primarily promote gene transcription changes. Also shown, is the ligand-independent GR signaling, which regulates gene transcription in the absence of GC ligand.

1.1.2.4 *A plethora of GR variants*

The main transcript derived from the GR gene is the GRα, representing the classic GR receptor protein, with a length of 777 amino acids (aa). Alternative splicing of exon 9, gives rise to GRβ, consisting only by 742 aa. In contrast to GRα, the GRβ isoform, is localized in the nucleus constitutively. When the two isoforms are colocalized, the GRβ form antagonizes GRα action by forming heterodimers that cannot induce transcriptional activation (Oakley and Cidlowski, 2013). Interestingly, the GRβ isoform can function in signaling pathways that are independent of GCs (Stechschulte et al., 2014). Alternative splicing in other exons generates three additional main isoforms GRγ, A and P. Furthermore, alternative translation initiation sites from the GRα mRNA give rise to different subtypes of GRα {-A, -B, -C1-3, -D1-3}, each one with distinct transcriptional profiles. Most notable are the GRα-D variants that are constitutively located in the nucleus and can regulate gene expression independent of GCs. During human brain development and in aging, the expression of GRα variants changes (Sinclair et al., 2011). Intriguingly the D variant was found to be increased in the brain in cases of neuropsychiatric disorders such as schizophrenia implicating altered GR signaling in the pathogenesis of a number of brain disorders (Oakley and Cidlowski, 2013).

Another level of regulation of GRs' function occurs at the post-translational level. Specifically, in response to GC binding the GR can be modified by phosphorylation, ubiquitylation, SUMO-ylation or acetylation. Each of these post-translational modifications alters the ability of the receptor to act on gene targets (Oakley and Cidlowski, 2013). Phosphorylation is the most common and most widely characterized of the modifications of the receptor and takes place on serine, threonine, or tyrosine residues located in the NTD. MAPKs, glycogen synthase kinase 3β and cyclin dependent kinases are amongst the most common

kinases that phosphorylate GR in response to GC stimulation. Consequences of phosphorylation can range from alteration of the transcriptional activity of the receptor, changes in cofactor recruitment, degradation of the GR or changes in the subcellular distribution of the receptor. Interestingly, phosphorylation of the human GR has been shown on Ser134, as a result of cellular stress, but in hormone free conditions, suggesting a role for GR in mediating the effects of stressful stimuli. For an in detail analysis of the GR receptor biology and signaling the reader is directed to the in depth reviews by Galliher-Beckley and Cidlowski (2009) and Oakley and Cidlowski (2013).

1.2 GC USE DURING PREGNANCY

1.2.1 *Synthetic GCs*

GCs have a plethora of actions and target tissues and are amongst the most prescribed medications in world. Because the endogenous GC form cortisol can be degraded from 11βHSD2, several synthetic forms of GCs (sGC) have been developed that are resistant to 11βHSD2 degradation such as BETA and DEX. The majority of total cortisol (95%), is bound to plasma proteins, ~77% is bound on corticosteroid binding globulin (CBG) and ~ 12% on albumin (Tegethoff et al., 2009). sGCs are more potent and have greater biological activities compared to cortisol, because they have a lower binding affinity to plasma proteins (Table 1.1). *Biological half-life* is defined as the duration of measurable biological activity. Both DEX and BETA are long acting GCs, with a biological half-life ranging from 36-54 hours, compared to cortisol which is short acting and has a biological half-life between 8-12 hours (Tegethoff et al., 2009). Moreover, the affinity and efficacy of sGCs in activating the genomic actions of GR are also higher compared to cortisol. BETA and DEX have a 25-fold higher potency in activating genomic GR signaling (BETA is slightly more potent than DEX). Also, sGCs have greater potency in activating non-genomic GR signaling compared to cortisol, with DEX being more than 5 fold higher of BETA (Grossmann et al., 2004; Tegethoff et al., 2009).

Table 1. Pharmacokinetic characteristics of Cortisol, Dexamethasone and Betamethasone in Humans

Glucocorticoid	Biological Half Life	Binding to Plasma Proteins
Endogenous Cortisol	Short Acting 8-12 hours	95% bound on plasma proteins (PPs): ~77% Albumin ~12 % CBG
Synthetic Dexamethasone	Long Acting 36 -54 hours	~75% PPs
Betamethasone		~60 % PPs

1.2.2 *Use of sGCs in prenatal medicine*

Glucocorticoids have been prescribed for use during pregnancy for many reasons including, maternal allergies and asthma. Synthetic GCs are also administered early in pregnancy when the fetus is suspected to suffer from congenital adrenal hyperplasia, an autosomal recessive condition resulting from mutations in the steroidogenic enzymes producing cortisol from cholesterol. This condition can affect both females and males and in its severe form, although not as common, results in virilization of the genitalia in females due to excess androgen production (Khalid et al., 2012). Due to the distress in later life associated with the ambiguous genitalia in females, DEX administration from the first trimester has been used as a treatment. Nevertheless, the malleability of this developmental period and the potential side-effects of DEX on embryo development have put this treatment under scrutiny. Another –unfortunately- common condition in which GCs are used, is preterm labor. Preterm labor is defined as birth before 37 weeks of gestation, with the normal range of human pregnancy spanning from 38-42 weeks.

Unfortunately, as the *Born2Soon Global report* [3] documented in 2012, more than 15 million babies are born prematurely each year, with over a million of babies dying as a result of complications from preterm birth (Lancet, 2012). Amongst the most common complications are intraventricular hemorrhage (IVH), as a result of an immature vasculature of the germinal matrix and development of respiratory distress syndrome (RDS) due to immaturity of the lungs. The germinal matrix is a proliferative zone of the human brain, which gives rise to neurons and glia, which migrate towards the developing cortex. It is highly vascularized, and the vasculature network is very delicate and thus highly sensitive to perturbations. The respiratory system matures during late gestation, during which type II pneumonocytes produce and secrete surfactant that allows the expansion of the lungs and prevents atelectasis of the lungs during breathing. In babies born before 37 weeks the pulmonary surfactant production is non-adequate or immature, and thus these babies have to spend the first stages of their life on a ventilator and supplied with exogenous surfactant. sGCs have been shown to reduce the mortality and morbidity associated with preterm birth by reducing the incidence of IVH and RDS. The mechanism of IVH reduction is not entirely clear but a stabilization of the germinal vasculature may be part of how sGCs act. In the case of RDS, sGCs reduce its incidence and the time preterm babies remain attached to a ventilator by promoting lung maturation and lung surfactant production.

[3] WHO | Born Too Soon: The Global Action Report on Preterm Birth
http://www.who.int/pmnch/media/news/2012/preterm_birth_report/en/

1.2.3 *Use of sGCs for premature labor: Historical Perspective*

The use of sGCs for the treatment of adversities resulting from premature labor, was pioneered by Sir Graham Collingwood Liggins (Figure 5). Dr. Liggins had devoted a great part of his research attempting to discover the trigger of premature birth using sheep as a model since many features of their pulmonary development is similar to humans. His observations that *in utero* exposure of premature lambs to GCs reduced the incidence and the severity of RDS (Liggins, 1969) lead to the seminal randomized and controlled-study in humans, where along with his colleague Dr. Ross Howie they showed that antenatal BETA can lead to a 2-fold reduction in RDS and a 5-fold reduction in the mortality of premature babies (Liggins and Howie, 1972).

Figure 5. Sir Graham (Mont) Liggins

Dr Liggins pioneered the use of synthetic glucocorticoids for the management of preterm labor saving the life of many preterm babies (Source: MacDonald, N., Dominion Post 2010 (MacDonald 2010).

1.2.4 *Current status of sGC use during pregnancy*

Following the seminal discoveries of Dr. Liggins, sGCs have been adopted as a standard treatment for the management of preterm labor, with DEX and BETA being the most common forms used (Braun et al., 2013; Lancet, 2012; Tegethoff et al., 2009). The current dose regime to avoid premature labor complications between weeks 24-34, is a dose of 24 mg given intramuscularly. DE is given as 4 doses of 6 mg every 12 hours, whereas BETA is given in 2 doses of 12 mg, 24 hours apart. As of 2013, the 18th World Health Organization model list of essential medicine, only includes DEX for the management of premature labor[4]. Depending on the development of the pregnancy, i.e., if preterm labor does not take place, more than one course of sGCs may be given.

Nevertheless, sGC treatment has limitations on its effectiveness. Specifically, sGCs are most efficient in reducing RDS and IVH when given between 26-34 weeks and these benefits are limited for a time of 1-7 days following the first dose. The benefits before 26 weeks are not clear, whereas after 34 weeks there are questions whether the benefits outweigh the potential neurological side-effects on the developing brain (Roberts and Dalziel, 2006).

[4] 18th WHO List of Essential Medicines, April 2013

1.3 EFFECTS OF GCS ON THE DEVELOPING BRAIN

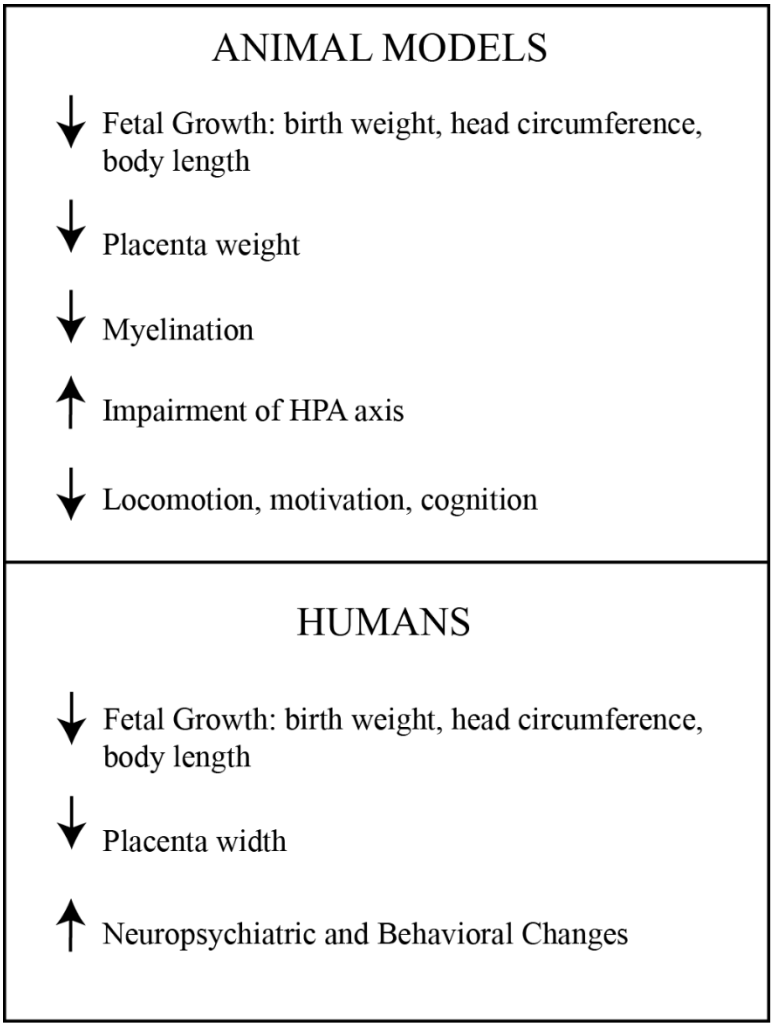
Despite their benefits in reducing the mortality and morbidity associated with preterm labor, antenatal exposure to sGCs can damage developing fetal organs including the brain. Accumulating evidence from human and animal studies support the hypothesis that *in utero* exposure to sGCs may affect the functioning of the HPA axis, providing a basis for altered endocrine system function in the offspring, which is then associated with an increased risk for metabolic, cardiovascular problems in later life. Also, due to the intimate link of the HPA axis to the hippocampal and other limbic areas including the prefrontal cortex, excessive levels of GCs, including those resulting from maternal stress, increase the risk for affective and neuropsychiatric disorders (Huang, 2011; Lupien et al., 2009).

1.3.1 *Animal Studies*

Due to the ethical constraints associated with human studies, animal models have provided valuable information in delineating the effects of GCs on the developing brain. Early studies done in the late 80s, when the use of sGCs started to increase in clinical settings, revealed that DEX exposure *in utero* can impair cortical and hippocampal development (Uno et al., 1990; Uno et al., 1994). Specifically, in rhesus monkeys exposed to DEX one month before birth, the cortical and hippocampal structures were hypoplastic, the pyramidal layers of the hippocampus severely underdeveloped and the cell somata were smaller. Moreover, the effect of DEX on the hippocampus was long-lasting since at 9 months of age the hippocampal volume was still smaller in the DEX-treated animals (Uno et al., 1990). Additional effects that prenatal sGCs may have include retardation of brain development (Carlos et al., 1992; Huang et al., 1999) and apoptosis

and reduction in the size of the postmitotic neurons (Kreider et al., 2006). Huang et al. (1999) evaluated the effects of a single versus multiple doses of BETA, on brain development in sheep. The effects on brain growth were evident even with the single dose, resulting in a reduction in cerebral length and depth and also to a reduction in whole brain weight at birth. Moreover, following antenatal sGC treatment in mice there have been reports of delayed neuronal migration in the cortex (Fukumoto et al., 2009), decreased proliferation of the hippocampus, accompanied by reduced LTD, impaired spatial learning, and reduction in the lifespan of the animals (Khozhai and Otellin, 2008; Noorlander et al., 2006; Noorlander et al., 2008). The same group reported a transient effect of DEX in increasing hippocampal apoptosis and reduction of the proliferation in the subgranular zone of the dentate gyrus upon birth, which was not observed in adult animals (Noorlander et al., 2014). In addition, Theodore Slotkin's group has demonstrated that even when rats are exposed *in utero* at doses of DEX well below the clinically relevant range, there are still long-lasting changes in brain development including growth inhibition and neuronal loss, alterations in synaptic activity and cell signaling, specifically in adenylyl cyclase (AC) in the cerebral cortex, hippocampus, and the striatum (Kreider et al., 2006). Finally, perinatal DEX exposure can stunt cerebellar development and cause apoptosis in the external granular layer (Heine and Rowitch, 2009; Heine et al., 2010; Noguchi, 2014).

Table 2. Summary of the effects of *in utero* GC exposure on various aspect of fetal growth and development, including of neural development in humans and animal models. Adapted from Braun et al. (2013).



1.3.2 *Human Studies*

The effects of antenatal GCs on human brain development have been the debate of many studies mainly due to the fact that it is difficult to achieve a randomized controlled study of this kind. Nevertheless, accumulating data from clinical studies are also pointing to an ambiguous role of antenatal GCs on brain development and putatively long term effects on the physiology of the offspring. Evaluation of the persons that were part of the original Liggins cohort 30 years later, revealed that adults exposed to a single dose of BETA had developed insulin resistance (Dalziel et al., 2005). Murphy et al. (2012), showed that multiple courses of antenatal GCs are associated with a reduction in birth weight and head circumference even after controlling for gestational age at birth. In addition, the authors noted that there was a trend for each additional course of GCs to correlate with incremental decreases in birth weight and head circumference. Normal variations in birth weight are strongly associated with cortical surface area and total brain volume, and thus correlates with later neural development (Walhovd et al., 2012). Within this context, repeated courses of GCs have been associated with delayed development and adverse mental health in childhood and adolescence (Khalife et al., 2013). Premature birth is itself associated with a number of complications that may impinge on brain development thus confounding any effects on brain development that can be attributed solely to antenatal GC exposure. In recognition of this fact, studies have aimed at collecting available data from children that were exposed to GCs antenatally, but were born at term (Waffarn and Davis, 2012). At this point it is important to note that 25-30% of women treated with GCs in the threat of an imminent premature labor do in fact continue at term (Davis et al., 2009). Alexander et al. (2012), evaluated cortisol responses in acute psychosocial stress in 6-11 year olds, exposed to prenatal GCs, but born at term. The results from this study not only revealed higher cortisol responses in the GC group indicating

long-lasting effects of the GC treatment, but also showed that the cortisol response more pronounced in females, highlighting a sex-specific effect of GCs on modifying the HPA axis (Harris and Seckl, 2011). Effects on brain development can be direct as a result in changes in the neurogenetic process or due to a reprogramming of the HPA axis which in turn affects key developmental processes including neurological maturation. Additionally, full term-born children (>37 weeks), exposed prenatally to a single course of BETA, were shown to have a reduced birth weight and also reduced head circumference compared to untreated controls (Davis et al., 2009). Importantly a recent study used structural magnetic resonance imaging (MRI) to evaluate different parameters of cortical development in school age children, which were exposed prenatally to GCs and born at term. Interestingly, the results revealed that the GC-treated group had significant widespread and bilateral cortical thinning. The area mostly affected was the anterior cingulate cortex (Davis et al., 2013), which is densely populated with GRs and it is tightly related to the pathophysiology of neuropsychiatric and affective disorders (McGowan et al., 2009). Along these lines, cortical thinning of the GC-group was associated with development of affective problems later in life (Davis et al., 2013).

Collectively the animal and human results point to adverse effects of prenatal GCs on brain development. Coming to add to this hypothesis are results from studies on neural stem cells in culture either of animal or human origin. Specifically, many groups have shown that GCs and specifically DEX which is the focus of this study, can impact multiple stages of neuronal development such as the proliferation of neural stem cells and cell cycle progression, cell fate and survival (Anacker et al., 2013; Peffer et al., 2014; Samarasinghe et al., 2011).

1.4 NEUROGENESIS

The cerebral cortex is the ultimate locus of human cognition, holding the secrets of reasoning, intellect, memory and imagination. The process by which the cortex develops, corticogenesis or neurogenesis, is a highly complex sequence of events, tightly controlled at multiple levels of organization, both at the temporal and spatial scale. Many of the insights we now have about the process of neurogenesis are due to the pioneering work of early developmental biologists such as Pasco Rakic, Joseph Altman[5] and Shirley A. Bayer. Altman and Bayer, mapped the developing brain using birthdating methods and created histological maps that remain valid today[6]. Pasco Rakics' experiments in monkeys have been instrumental in understanding how embryonic neurogenesis in primates proceeds and generates the elaborate cortical structure of the adult organism (Rakic, 1988). Moreover, pioneering work by Verne S. Caviness, Jr, Richard S. Nowakowski, Tsutomu Takahashi and Robert F. Hevner in the early 90s established the foundations for understanding how neuronogenesis takes place from progenitors in the neural epithelium of the embryonic mouse. The aforementioned scientists are only a handful of the key figures in the arsenal of the neural development area. The field of cortical development is a constantly expanding galaxy of discoveries, since the complexity of the processes that govern neurogenesis are still poorly understood and still hold many secrets. In this section we provide a brief overview of the basic events and key players in the cortical development of the mouse, which is the model we use in this study. The reader is referred to the excellent reviews by Kohwi

[5] Joseph Altman was the first to show that neurogenesis also takes place in the adult brain, with the discovery of the rostral migratory stream (Altman, 1969).

[6] The complete series of studies by Altman and Bayer is available online at: *http://neurondevelopment.org/*

and Doe (2013); Sun and Hevner (2014) and Greig et al. (2013), for an in depth and detailed update on the topic of neural development.

1.4.1 *The neurogenetic process*

The cerebral cortex develops from the rostral most part of the neural tube from neuroepithelial stem cells (NEs) lining the surface of the cerebral epithelium. These neuroepithelial cells give rise to neural progenitor cells which initiate neurogenic divisions around embryonic day 10.5 (E10.5) in the mouse. Neuronal production progresses from E11.5 until early E17.5, during which the last neurogenic divisions take place (Takahashi et al., 1999). Between E11.5-E17.5, neural progenitors divide going through successive cycles of divisions that culminate in a total number of 11 complete cell cycles. The cell cycle duration increases with progression of neurogenesis, mainly due to an increase in G1 phase, starting with a duration of around 8 hours on E11.5 which increases to 18 hours on E16.5 (Takahashi et al.,(Miyama et al., 2001; Takahashi et al., 1994; Takahashi et al., 1995; Takahashi et al., 1997). At the beginning of neurogenesis the majority of divisions are proliferative and aim in expanding the progenitor pool (Proliferative fraction, P, ~1), whereas only a small proportion of divisions, generate neurons (Quitting fraction, Q). With the progression of neurogenesis, the rate of proliferation comes to an equilibrium with the rate of neuronal production during midgestation (E14.5, P=Q=0.5), whereas at the end of neurogenesis Q is ~ 1 (Takahashi et al., 1995; Takahashi et al., 1999). Beyond the temporal scale, neurogenesis is also controlled at the spatial scale, with rostral and ventral areas of the cortex pacing ahead of caudal and dorsal areas in terms of the time of neuronal production initiation (Caviness et al., 2009; Takahashi et al., 1997).

1.4.2 *Neural progenitors*

The main proliferative population of the neuroepithelium are radial glia cells (RGCs) which are progenitor cells derived from the initial population of neuroepithelial stem cells. RGCs reside in the ventricular zone (VZ) and are characterized by the expression of specific transcription factors such as the nuclear Paired box protein 6 (Pax6), the expression of which is usually used to characterize the VZ outline. RGCs, have elongated polarized processes, one on the apical side of the ventricular zone and the other impinging on the pial surface (Sun and Hevner, 2014) (Figure 6B). Through early and past mid-neurogenesis RGCs generate neurons, whereas around E16 and onwards, these cells undergo a competence change and start giving rise first to astrocytes and then to oligodendrocytes (Campbell and Gotz, 2002; Rakic, 2007; Rakic, 2009). Nascent neurons use the radial glia processes as guiding scaffolds to migrate to the cortical plate. With the temporal progression of neurogenesis, later born neurons of the upper layers migrate through the established early born, deep layer neurons eventually forming the typical six-layered cortical plate in an inside-out manner (Greig et al., 2013).

During the neurogenic phase, RGCs mainly undergo asymmetric divisions and give rise to one RGC and one neuron or one intermediate progenitor cell (IPC), (Englund et al., 2005; Kohwi and Doe, 2013; Sun and Hevner, 2014). The intermediate progenitor cells (IPCs), form the subventricular progenitor zone (SVZ), consist the second progenitor population of the cortex and they are characterized by the expression of T-box brain protein 2 (Tbr2) in the nucleus (Figure 6C). In contrast to the RGCs which can go through many rounds of division expanding their numbers, IPCs are primarily neurogenic with a limited self-renewal capacity. Accordingly, IPCs can go two or three rounds of self-renewal divisions, but usually they divide symmetrically to two neurons (Pontious et al., 2008).

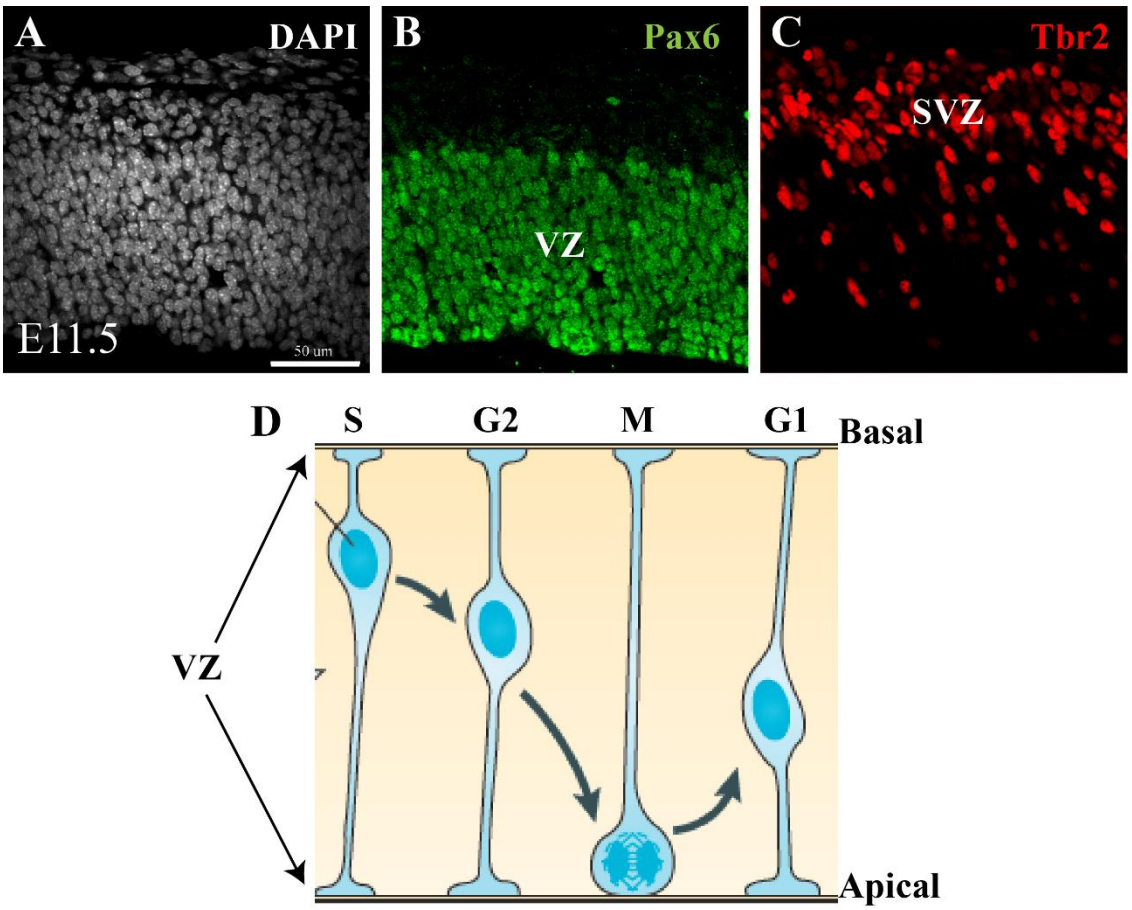


Figure 6. Cortical progenitors and Interkinetic Nuclear Migration

A-C. The neuroepithelium consists of two types of progenitors, (B) Radial glia cells (RGCs) which specifically express Pax6 (green) and (C), Intermediate progenitors (IPCs) which are specifically marked with Tbr2 (red). RGCs form the ventricular progenitor zone (VZ) can go many rounds of self-renewal and give upon commitment can give rise to IPCs or neurons. IPCs form the subventricular progenitor zone (SVZ) are neurogenic and only go two-three divisions before generating two neurons. ***D.*** RGCs undergo interekinetic nuclear migration (INM), whereby the nucleus of the cell completes S phase in the basal VZ and then moves towards the apical VZ through G2 and completes M phase on the apical surface. Then it translocates towards the basal side again through G1. (D) is adapted from Sun and Hevner, 2014.

1.4.3 *Molecular mechanisms controlling cortical development*

The process by which the cortex is built starting from a simple pseudoepithelium to its mature form, is highly complicated. The mature cortex comprises by constellations of different cell types that form complex neuronal circuits, which are connected with the most intricate patterns to achieve maximum precision in information flow within and out of the cortex. To achieve this architectural wonder, neurogenesis is highly regulated in the spatial and temporal axis, with neuronal production along the rostro-lateral axis preceding neuronal production on the dorso-caudal axis. Moreover, the fate of the progenitors is regulated on a temporal scale. Specifically, cell intrinsic or extrinsic signals impinge upon the progenitors during specific plasticity windows to alter the competence of the neural progenitors and thus affect their fate (Kohwi and Doe, 2013). An example is that deep layer neurons are generated during the early stages of neurogenesis whereas upper layer neurons, are generated from mid-neurogenesis and onwards (Greig et al., 2013).

1.4.3.1 *Control of cell cycle dynamics and symmetric versus asymmetric divisions*

The cell cycle is a critical factor of progenitor fate since it has been observed from early studies (Takahashi et al., 1994; Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1999) that the cell cycle length increases as neurogenesis progresses in parallel with the increase in neuronal output. Neurogenic RGCs and IPCs can be distinguished from their counterparts that are undergoing proliferative divisions by the expression of the Tis21 protein (Iacopetti et al., 1999). Using Tis21-GFP knock-in mouse embryos, RGC- and IPC-specific markers, Arai et al. (2011) demonstrated by birthdating that neurogenic progenitors have longer cell cycles than self-expanding progenitors. This happens due to an increase of the G1 phase and shortening of the S

phase. Moreover, they showed that in general IPCs have longer cell cycles than RGCs, with 26.5 hours versus 19.1 hours respectively. Hence, cell cycle dynamics are critical for cell fate and differ within and between progenitor types depending on the competence of the cell (i.e., self-renewing versus neurogenic). Moreover, RGCs and apical progenitors throughout the cortex in general, have a characteristic pattern of cell cycle progression (Pilz et al., 2013). The nucleus of the cells goes through S phase in the basal aspect of the VZ and then migrates towards the apical VZ through G2 to eventually complete mitosis (M phase) on the apical surface (Figure 6D). Then it migrates again towards the basal VZ through G1 phase. This phenomenon is called *Interkinetic Nuclear Migration* (INM). Factors that disrupt INM, also affect the fate of RGCs (Buchman and Tsai, 2008; Kageyama et al., 2009; Latasa et al., 2009; Murciano et al., 2002; Willardsen and Link, 2011). For example, conditional deletion of the proliferation regulator, forkhead transcription factor M1 (Foxm1), which is expressed in RGCs but not IPCs, alters the synchronization of RGCs and promotes the IP fate (Wu et al., 2014).

Additionally, Pax6 itself is a master player in cell cycle progression and regulator of the switch from proliferation to neuronogenesis (Kohwi and Doe, 2013). Loss of function of Pax6 results in the RGCs dividing in basal locations, and in an increase in S-phase duration which results in smaller cortices due to a compromise in the progenitor pool (Estivill-Torrus et al., 2002). Interestingly, in Pax6 null cortices, loss of Pax6 function has biphasic effects depending on the stage of neurogenesis. Specifically, from early until mid-neurogenesis, Pax6 loss initially lead to a decrease in cell cycle duration, increase of asymmetric divisions and precocious onset of neurogenesis. Furthermore, from mid-neurogenesis the length of the cell cycle increased due to a prolongation in S phase (Estivill-Torrus et al., 2002). These results could be explained partly due to the fact that Pax6 exerts regulatory control of the G1 to S phase transition by suppressing

cyclin dependent-kinase (CDKs) and cyclin signaling such as CDK6, Cyclin1 and 2. This has as a result the repression Rb phosphorylation, which normally promotes cell cycle progression and DNA replication (Mi et al., 2013).

Collectively, the fate of proliferating progenitors is highly dependent on the cell cycle dynamics. A depletion of the progenitor pool due to a favoring of asymmetrical over symmetric RGC divisions or reduction in the progenitor proliferation in general (RGCs and IPCS), ultimately will lead to a smaller neuronal output and a smaller cortex.

1.5 OBJECTIVES

This study is fuelled by the combined fact that antenatal use of GCs can lead to detrimental effects on neurogenesis, cortical development and the offspring behavior. Yet, the exact role of the GR in neural progenitors and in neurogenesis has not been explored in detail. Therefore in this study we sought to explore two objectives using the mouse embryo as a model:

Objective 1: Map the temporal and spatial expression of the GR protein in the RGCs and IPs of the developing telencephalon, in vivo.

To our knowledge, a detailed study documenting the spatial and temporal expression of the GR protein in the RGCs and IPCs during the time window that neurogenesis is taking place, is currently lacking. This applies both for animal models and the human, in which case the results from animal models would need to be translated ultimately. Interestingly for the human embryo, GR mRNA was detected as early as the first trimester (Condon et al., 1998; Costa et al., 1996). In the case of the mouse embryo, GR mRNA was detected in the brain from around E12 (Speirs et al., 2004). Nevertheless, neither of these studies focused specifically on the brain nor did they examine GR protein expression in progenitors. Considering that progenitor fate and progenitor competence varies according to the developmental stage, knowledge of GR's ontogeny is critical to our understanding of how and why antenatal GCs lead to differential effects on the developing brain according to the time of exposure (see section 1.3). Therefore the first part of this study we sought to map the developmental profile of the GR protein expression in the embryonic mouse telencephalon in RGCs and IPCs during the neurogenic window i.e., E11.5-E17.5. We predicted that GR protein will be present in progenitors from early neurogenesis.

Objective 2: Dissect the effects of premature DEX-induced GR activation on the properties of neural progenitor cells and cytoarchitecture of the developing cortex.

During the time window that DEX is used for the management of premature labor, i.e., 24-34 weeks of gestational age, the human brain is still developing (Kapellou et al., 2006). Specifically, the critical window for the development of cortical convolutions lies between 26–36 weeks (Dubois et al., 2008), and thus coincides with the window that DEX is used (Figure 7). As described in Section 1.3, the effects of prenatal DEX can be variable depending on the dosing regimen used in the study and additionally, on the number of courses used. Moreover, a fact that to our knowledge has not been highlighted in previous studies, is that during the time that babies are exposed to DEX, the endogenous levels of GCs are relatively low and therefore the activation of GR from endogenous GCs is expected to be low. The embryo starts to produce cortisol in increased quantities during late gestation in preparation for labor while the maternal contribution is minimal due to degradation of maternal cortisol by the placental 11βHSD2 (see Section. 1.1.1.1). Consequently, exposure of the embryo to DEX will result in a premature activation of the GR signaling and potentially may disrupt any role that the unliganded GR may have during neurogenesis. Therefore in this aim we sought to dissect the effects of a single clinically relevant course of DEX on the properties of neural progenitors during mid-gestation (E14.5) in the mouse embryo hypothesizing that premature Dex-induced GR signaling will disrupt the proper course of neurogenesis by affecting the properties of neural progenitors. Since GC production in the mouse embryo initiates around E16, this experimental scheme provides a GC naïve environment to explore the effects of premature DEX-induced GR signaling on neurogenesis while being as close as possible to the neurodevelopmental effects of the human brain.

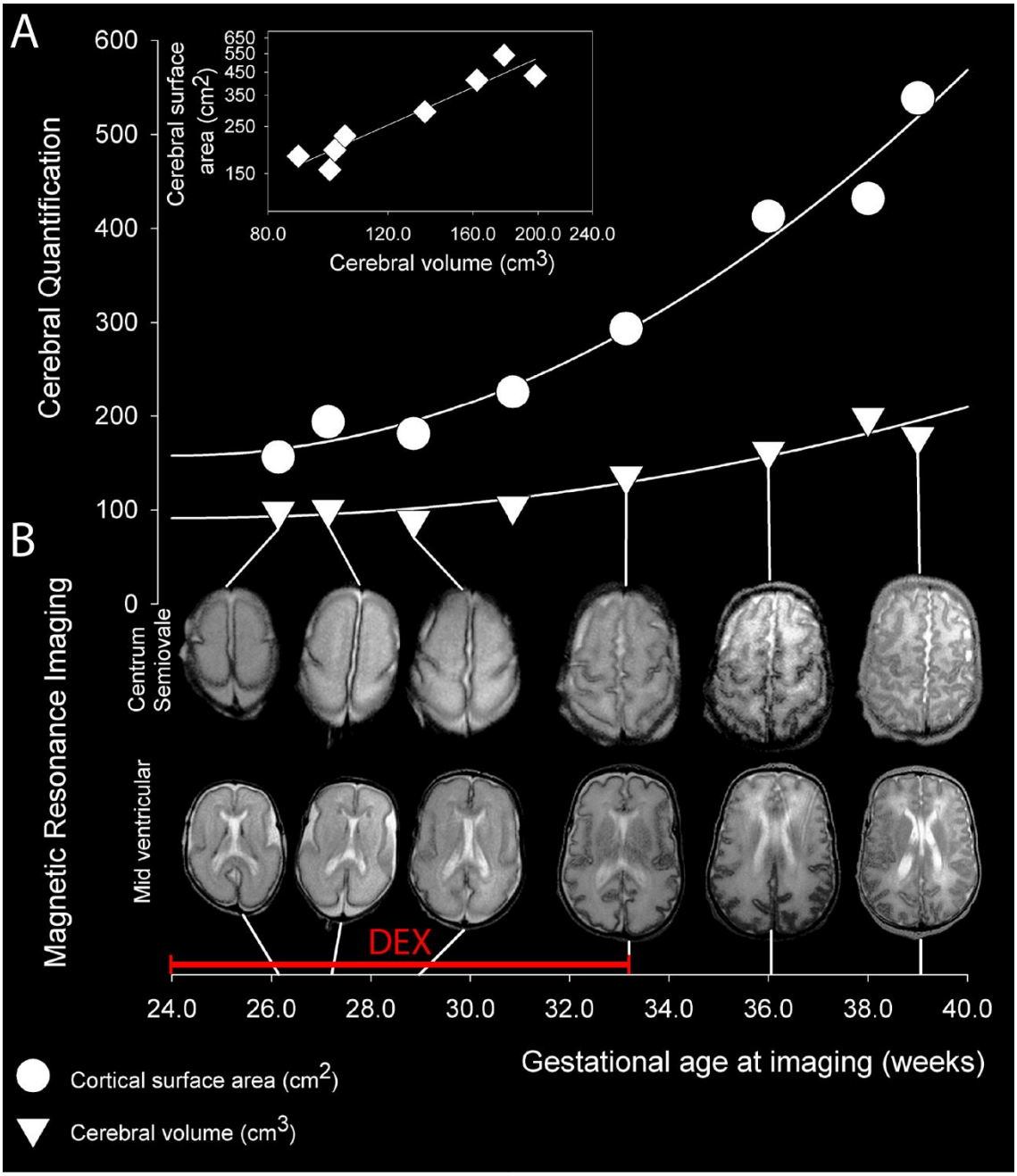


Figure 7. Timeline of human cortical development from 24 weeks of gestational age until birth.

A. The cortical surface rapidly increases from 30 weeks and onward**,** whereas, ***B.*** critical cortical convolutions arise between 26-36 weeks of gestation, coinciding with the time DEX is prescribed for preterm labor. Adapted from and **Copyright** © of Kapellou et al. (2006). Material used under the terms of the Creative Commons Attribution License.

2.0 FIRST CHAPTER

DIFFERENTIAL SUBCELLULAR LOCALIZATION OF THE GLUCOCORTICOID RECEPTOR IN DISTINCT NEURAL STEM AND PROGENITOR POPULATIONS OF THE MOUSE TELENCEPHALON *IN VIVO*

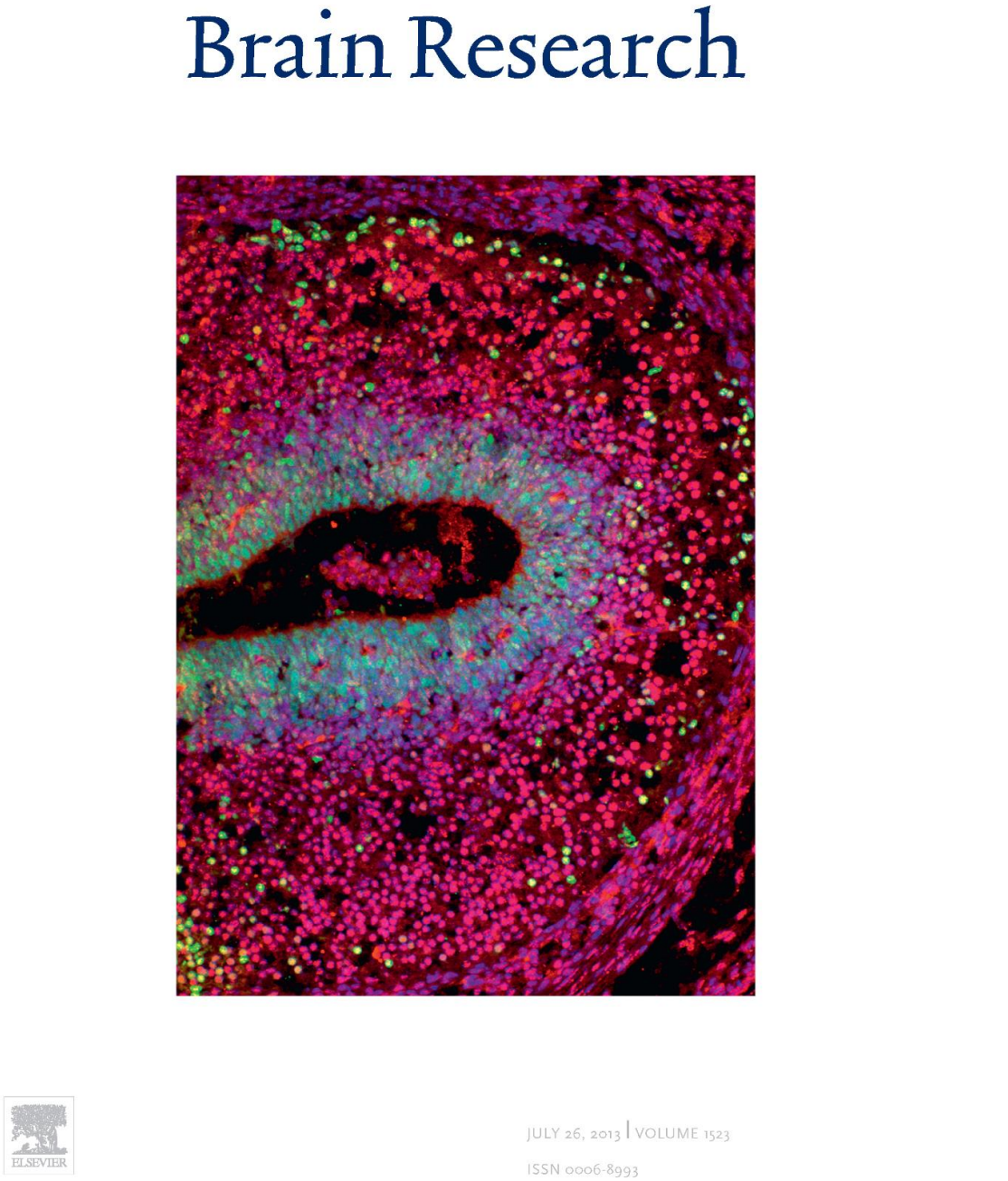


Figure 8. Tsiarli et al., 2013, Issue Cover

2.1 INTRODUCTION

Preface

Glucocorticoids are given to pregnant women at risk for premature delivery to promote lung maturation. Despite reports of detrimental effects of glucocorticoids on telencephalic neural stem/progenitor cells (NSPCs), the regional and cellular expression of the glucocorticoid receptor (GR) in various NSPC populations in the intact brain has not been thoroughly assessed. Therefore in this study we performed a detailed analysis of GR protein expression in the developing mouse ventral and dorsal telencephalon *in vivo*. At embryonic day 11.5 (E11.5), the majority of Pax6-positive radial glial cells (RGCs) and Tbr2-positive intermediate progenitor cells (IPCs) expressed nuclear GR, while a small number of RGCs on the apical ventricular zone (aVZ), expressed cytoplasmic GR. However, on E13.5, the latter population of RGCs increased in size, whereas abventricular NSPCs and especially neurons of the cortical plate, expressed nuclear GR. In IPCs, GR was always nuclear. A similar expression profile was observed throughout the ventral telencephalon, hippocampus and olfactory bulb, with NSPCs of the aVZ primarily expressing cytoplasmic GR, while abventricular NSPCs and mature cells primarily expressed nuclear GR. Close to birth, nuclear GR accumulated within specific cortical areas such as layer V, the subplate and CA1 area of the hippocampus. In summary, our data show that GR protein is present in early NSPCs of the dorsal and ventral telencephalon at E11.5 and primarily occupies the nucleus. Moreover, our study suggests that the subcellular localization of the receptor may be subjected to region and neurodevelopmental stage-specific regulation.

Keywords: glucocorticoid receptor, neural stem/progenitor cells, neurodevelopment, embryo, cortex

The results of this chapter have been published in *Brain Research,* 2013. The citation for the study follows. Figure 10A of the article (Figure 8 of this document), shown in the previous page, was selected for the issue cover.

Tsiarli, M.A., Paula Monaghan, A. & DeFranco, D.B.

Differential subcellular localization of the glucocorticoid receptor in distinct neural stem and progenitor populations of the mouse telencephalon *in vivo*. *Brain Res* 1523, 10-27 (2013).

In addition to their widespread use as anti-inflammatory agents in adults and children, glucocorticoid hormones (GCs) have become standard antenatal therapy to reduce complications of premature delivery since the early 1970s (Liggins, 1969; Liggins and Howie, 1972). GCs promote fetal lung maturation and are given to premature babies or to pregnant women at risk for premature delivery during late gestation. Synthetic GCs such as dexamethasone (DEX) are commonly used in this context and have been shown to reduce respiratory distress syndrome development in premature infants (Roberts and Dalziel, 2006). Moreover, DEX is used antenatally to reduce virilization of female fetuses suspected of having Congenital Adrenal Hyperplasia (Rivkees and Crawford, 2000). The intracellular effects of GCs are primarily mediated by the glucocorticoid receptor (GR), a member of the superfamily of nuclear receptors. These receptors function as ligand-regulated transcription factors and play important physiological roles in organogenesis, metabolism, homeostasis and stress responses (Tang et al., 2011).

Despite the benefits of prenatal GCs in reducing the development of respiratory distress syndrome and increasing preterm infant survival, accumulating evidence both from animal and clinical studies have associated prenatal GC exposure with detrimental effects on brain development. For example, *in utero* exposure to DEX can cause detrimental effects on brain morphology such as reduced size and altered layering both in the cortex and the hippocampus (Khozhai and Otellin, 2008; Uno et al., 1994). Moreover, prenatal GCs can disrupt the function of the hypothalamus-pituitary-adrenal (HPA) axis and thus permanently alter the stress response of the offspring (Huang, 2011). Likewise, clinical follow-up studies of children and adults exposed perinatally to high levels of GCs, reported cognitive and behavioral abnormalities (Lupien et al., 2009; McEwen, 2005; Purdy et al., 2008; Reynolds and Seckl, 2012; Welberg and

Seckl, 2001). Furthermore, there are numerous reports linking high levels of GCs *in utero* with cognitive abnormalities and even increased probability of developing schizophrenia (Bombin et al., 2012; Huang, 2011; Lupien et al., 2009). On a cellular level, GCs reduce the proliferation and alter cell cycle dynamics of both embryonic and adult neural stem/progenitor cells (NSPCs) (Heine and Rowitch, 2009; Heine et al., 2010; Kim et al., 2004). The observed effects on NSPC properties are the combinatorial result of the genomic actions of the nuclear ligand-bound GR and of non-genomic signaling initiated by GC activation of GRs bound on the plasma membrane (Samarasinghe et al., 2011). Despite the interest in the actions of GCs in the developing brain and their putative link to neuropsychiatric disorders, there is limited information regarding the distinct NSPC populations that express GR protein throughout fetal development. In the rat, GR mRNA expression was detected as early as E13 in the developing brain (Cintra et al., 1993; Kitraki et al., 1996), while in the mouse GR transcripts have been detected as early as E12 in the developing telencephalic neuroepithelium (Speirs et al., 2004). These studies however did not examine the temporal or cell type-specific expression of GR protein, or its subcellular localization. We therefore employed immunohistochemistry to define the spatial and temporal *in vivo* localization of the GR protein in distinct cellular populations of the developing mouse telencephalon. Specifically, we focused on the dorsal telencephalon, the future cerebral cortex, where cellular abnormalities associated with various neuropsychiatric disorders may first be manifested. Our study reveals that GR protein is present in early NSPCs of the dorsal and ventral telencephalon at E11.5 and primarily occupies the nucleus. Also, our data show that the subcellular localization of GR in NSPCs changes from E13.5 and on, suggesting that the subcellular localization of the receptor may be subjected to region and neurodevelopmental stage-specific regulation.

2.2 EXPRERIMENTAL PROCEDURES

2.2.1 Animals

Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC) and pregnant dams were housed individually and received chow and tap water *ad libitum*. The day of vaginal plug was designated as E0.5. Embryos were collected via caesarian section and were fixed in 4% paraformaldehyde (PFA; pH 7.4) and processed through increasing sucrose gradients for cryosectioning. Subsequently, brains were embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (EMS, Hatfield, PA) and sectioned at 20 µm. Animal protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and adhered to the National Institutes of Health guidelines. At least 8 animals were used in the E11.5, E13.5 groups and at least 3 animals for the E17.5 and P0.5 groups.

2.2.2 Immunohistochemistry

Immunohistochemistry for GR and other cell-markers were applied as described previously (Harrison et al., 2008). Briefly, cryosections were dried for 30 minutes at 46ºC or air-dried at room temperature for approximately an hour and then washed with 0.1% Triton-PBS. The blocking step was performed with 10% heat-inactivated normal goat serum (HINGS, Jackson ImmunoResearch, West Grove, PA), for 1 hour at room temperature, followed by three overnight incubations at 4°C with the primary antibody, which was diluted in blocking solution. Sections were subsequently incubated for 1 hour in the secondary antibody at room temperature, rinsed

and counterstained with 4,6-diamidino-2-phenylindole (DAPI). The following primary antibodies were used: mouse anti-BuGR2 (1:200, homemade), rabbit anti-Pax6 (1:500, Covance, Berkeley, CA), rabbit anti-Tbr2 (1:1000, Abcam, Cambridge, MA), rat anti-Ctip2 (1:500, Abcam), chicken anti-Tuj1 (1:200, Neuromics, USA), rabbit anti-Sox2 (1:1000, Abcam), rabbit anti-GABA (1:1000, Sigma-Aldrich ,St. Louis, MO), rabbit anti-CB (1:1000, Millipore, CA, USA). Secondary antibodies included anti-mouse AlexaFluor 555, anti-rabbit AlexaFluor 488, anti-chicken AlexaFluor 647 and anti-rat AlexaFluor 546 (Invitrogen, Carlsbad, CA). Fluorescent images were visualized on a Nikon fluorescent microscope, photographed with a Photometrics CoolSNAP digital camera and IP Lab software or with an Olympus Fluoview 1000 confocal microscope (single optical sections and z-stacks, 10X, 20X, 40X or 60X oil immersion, single optical section thickness 2.5 μm). Images were adjusted in Photoshop CS4.

2.3 RESULTS

2.3.1 The BuGR2 antibody specifically detects GR protein in the mouse telencephalon.

GR protein expression was examined in the telencephalon of mouse embryos from E11.5, a point when neurogenesis is at early stages (Gotz and Huttner, 2005). For all of the analysis performed in this study we utilized a concentrated preparation of the BuGR2 monoclonal antibody (Gametchu and Harrison, 1984), which was generated in our laboratory and has been used extensively on a variety of cell types (Qi et al., 1989; Samarasinghe et al., 2011). Moreover, BuGR2 has been validated as an appropriate GR antibody for in vivo immunohistochemical studies (Sarabdjitsingh et al., 2010) and has been used to examine GR expression in the adult hippocampus (Fitzsimons et al., 2008). In order to demonstrate the specificity of BuGR2 we examined the expression of GR protein within regions of the postnatal mouse brain, where GR expression and localization is well established (Usuku et al., 2005). As shown in Figure 1, the BuGR2 antibody detected GR in the Cornu Ammonis (CA) areas of the hippocampus, with the highest level of expression in the CA1 area and the subiculum (Fig. 9A), in agreement with previously published results. In addition, GR was detected in the Dentate Gyrus (DG) (Figs. 9A, B, F), as also shown by Patel and Bulloch (2003).

As additional validation, we compared the BuGR2 staining pattern of the cerebral cortex (Figs. 9A, C) and DG (Figs. 9A, B, F) to GR expression data from EGFP-Nr3C1 (GR) mice as published by the Gene Expression Nervous System Atlas Project (GENSAT) (Gong et al., 2003). As shown in Figure 9, the BuGR2 staining pattern in the cortex (Figs. 9A, C) and the DG (Figs. 9A, B, F) reproduced GR expression in the cortex and the DG of EGFP-Nr3C1 mice (Figs. 9D, E, G). Thus, these results show that the BuGR2 can specifically detect GR in histological

sections from the mouse telencephalon.

The GENSAT EGFP data in the postnatal cerebral cortex reveal the expression of GR in neurons of the cortex (Figs. 9D and 9E) and the hippocampus (Fig. 9F). However, since EGFP expression is driven by the GR promoter, it does not capture the detailed subcellular expression of GR as observed with the BuGR2 antibody. Specifically in our study immunohistochemical analysis of GR expression using the BuGR2 antibody revealed GR expression both in the cytoplasmic and nuclear compartments (Figs. 9A and Figs. 9G-H).

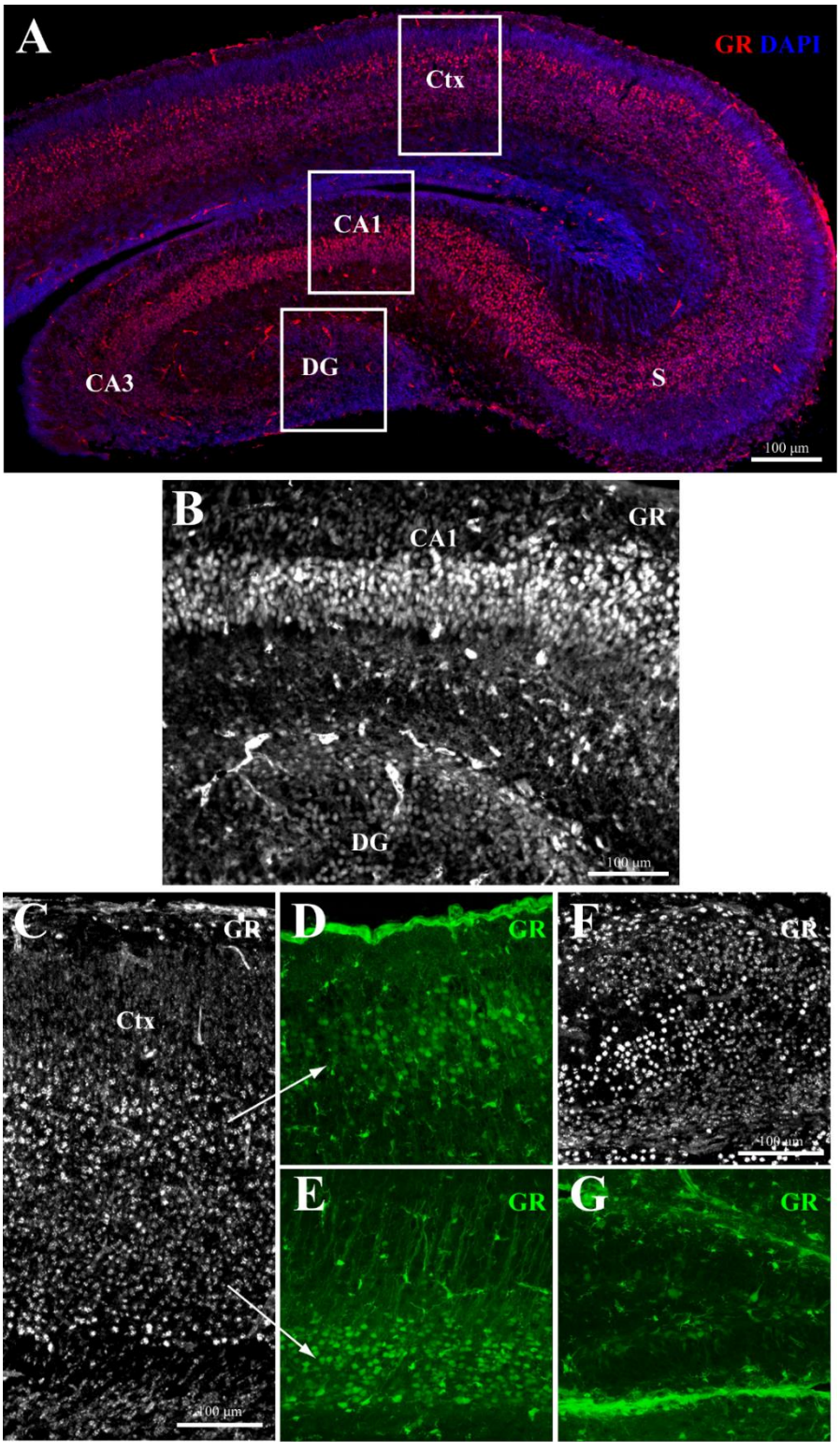


Figure 9. The BuGR2 antibody specifically detects the GR protein

in the postnatal cortex (A) and the hippocampus (A, B). The pattern of GR expression (red in A and white in the grayscale images in B,C,F) as detected by the BuGR2 antibody and visualized in single optical sections of the cortex (C) and the Dentate Gyrus (B, F, DG), is similar to the expression pattern of GR in the cortex (D, E) and the DG (G) of P7 Nr3c1(GR)-EGFP-reporter mice (Gong et al., 2003; Heintz, 2004). The images are courtesy of the Gene Expression Nervous System Atlas (25X, GENSAT, Rockefeller University, http://www.gensat.org/). CA, Area Cornus Ammonis; Ctx, cortex

2.3.2 Expression of the GR protein in the dorsal telencephalon

2.3.2.1 GR protein is widely expressed in the E11.5 embryonic body, with high levels of expression in the central nervous system.

GR mRNA has been detected from E9.5 with a peak of fetal expression by E11.5 in the mouse (Speirs et al., 2004). We similarly observed widespread expression of the GR protein in E11.5 mouse embryos (Fig. 10). In particular, non-neuronal areas with prominent GR expression included the lung, gastrointestinal track, blood vessels (Fig. 10A) and the developing spinal cord (Fig. 10B). In the embryonic central nervous system, GR was widely expressed. Relatively high levels of expression were observed in the developing cerebellum (Figs. 10A, C), hippocampus, (Figs. 10A), hypothalamus, pituitary and cranial nerves (Figs. 10A, D, E). Importantly, GR protein was detected in both the dorsal (future cerebral cortex) and ventral (ganglionic eminences) telencephalon (Fig. 10A). In most embryonic tissues at this age, GR appeared to be localized predominantly in the nucleus as evidenced by GR expression in the grayscale images 10B-E.

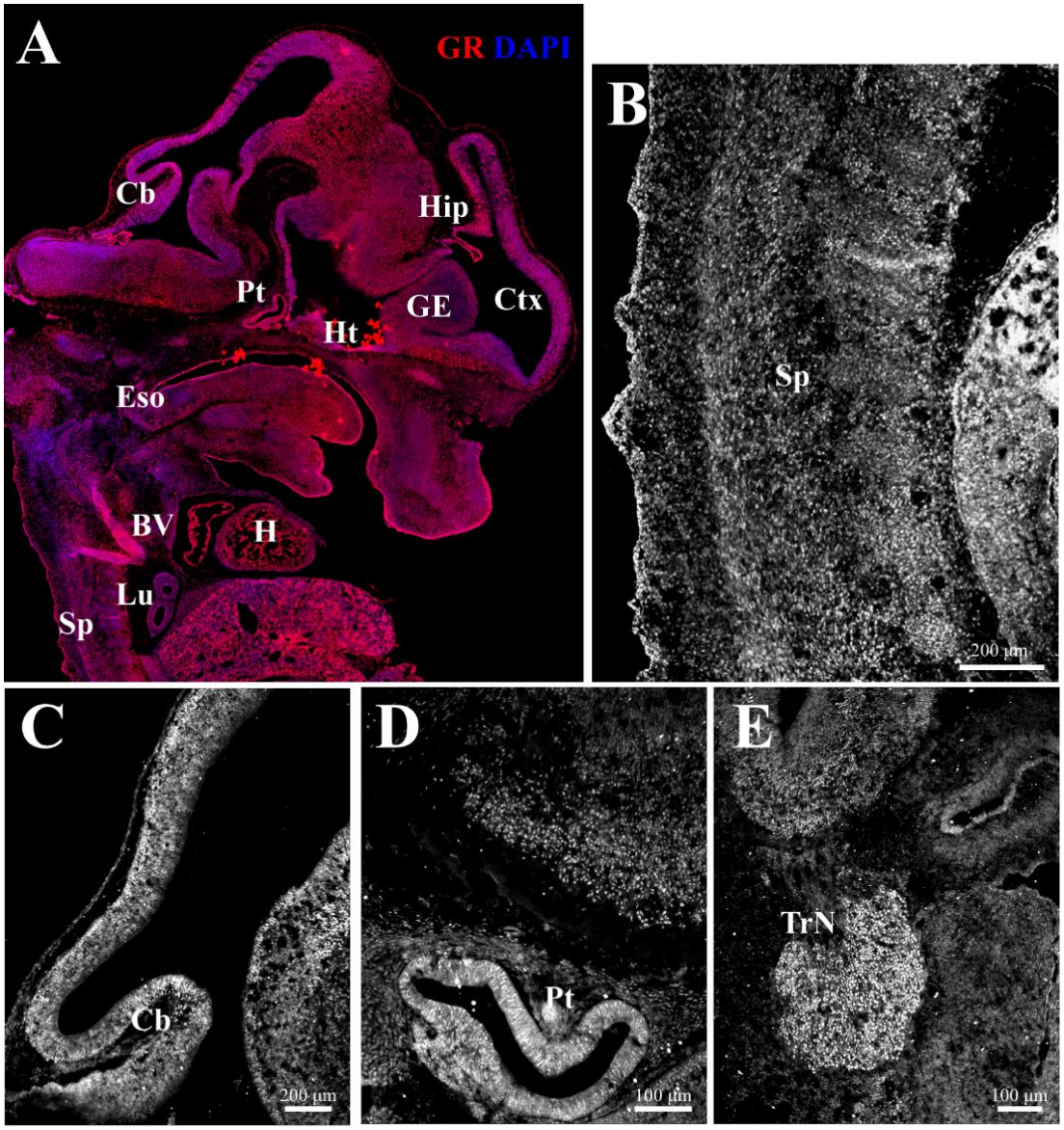


Figure 10. GR is ubiquitously expressed throughout the embryonic body at E11.5.

(A). Sagittal section throughout an E11.5 mouse embryo, stained with BuGR2 (red) and counterstained DAPI (blue). GR is ubiquitously expressed throughout the embryonic body including the spinal cord (A, B, Sp), with robust expression levels in some areas such as the cerebellum (C, Cb), the hypothalamus (A, Ht), the pituitary (D, Pt) and the trigeminal nucleus (E, TrN). The hippocampus (A, Hip) is one of the areas with the highest expression levels of GR in the central nervous system.Ctx, cortex; GE, ganglionic eminence; Lu, lung; Sp, spinal cord; H, heart; Eso, esophagus; BV, blood vessels

2.3.2.2 GR Protein is Expressed in Radial Glial Cells (RGCs), Intermediate Progenitor Cells (IPCs) and Neurons at E11.5.

In light of the behavioral and cognitive abnormalities observed in children exposed to GCs pre- or perinatally, we specifically focused on the developmental expression of GR in the dorsal telencephalon beginning on E11.5, an early neurogenesis stage. In the E11.5 dorsal telencephalic neuroepithelium, we observed variable GR expression and subcellular localization within distinct subregions and NSPC populations (Fig. 11A, B). Specifically, a graded pattern of GR protein expression was observed in NSPCs along the apical to basal extent of the neuroepithelium (i.e., from the ventricular zone to the pia surface). NSPCs in the apical ventricular zone (aVZ) exhibited relatively low to minimal levels of GR protein within their nucleus, while in some NSPCs GR was localized in the cytoplasm (Figs. 11C-F). In contrast, NSPCs located in the basal ventricular zone (bVZ) expressed relatively high levels of nuclear GR (Figs. 11C-F). Moreover, neurons in the preplate layer (PP) exhibited strong nuclear GR localization (Figs. 11C-F).

At E11.5, the dorsal telencephalon neuroepithelium mainly consists of RGCs, which undergo symmetric proliferative divisions and expand the progenitor cell pool. Subsequently, RGCs through asymmetric divisions to give rise IPCs and neurons, while IPCs mainly engage in terminal neurogenic divisions (Pontious et al., 2008). To determine the pattern of GR expression within each of these NSPC populations, we performed double indirect immunofluorescence (IIF) labeling with BuGR2 and the RGC-marker Pax6, or BuGR2 and the IPC-marker, Tbr2. While GR was expressed in both RGCs (Figs. 11A, D) and IPCs (Figs. 11B, E, F), the subcellular localization of the receptor differed between these two progenitor cell types. Specifically, most of the Pax6-positive RGCs in the aVZ expressed relatively low levels of GR in the nucleus, with more prominent cytoplasmic GR expression (Figs. 11C, D; confocal z-stacks provided in

Appendix A. Figs. 35A-C). In contrast, Pax6-positive RGCs in the bVZ expressed relatively high levels of nuclear GR (Figs. 11C, D). Nuclear colocalization of GR and Pax6 is most obvious by examination of the degree of overlap (yellow/orange) between the BuGR2 stain (red) and of Pax6 (green) in Figure 11D and in Sup. Figs. 1A, B. On the contrary, in Tbr2-positive IPCs GR was localized within the nucleus (Figs. 11E, F and Sup. Fig. 1D).

At early stages of neurogenesis, Tbr2 is also expressed in newborn neurons of the PP (Englund et al., 2005), as also shown in Figure 11F. Moreover, the PP also hosts pioneer neurons originating both from within and outside the dorsal telencephalon (Gotz and Huttner, 2005). Therefore, to distinguish between neurons and IPCs, we performed triple IIF for BuGR2, Tbr2 and the neuron specific marker, Tuj1. As shown in Figure 11F, both neurons (Tuj1-positive/Tbr2-positive cells, circles; Tuj1-positive/Tbr2-negative yellow arrows) at the bVZ/PP boundary and IPCs (Tuj1-negative/Tbr2-positive cells, white arrows) expressed nuclear GR (Appendix A. Figs. 35D-F). Therefore, in the E11.5 dorsal telencephalon, GR protein was present in the nucleus of the majority of RGCs, IPCs and in neurons of the PP.

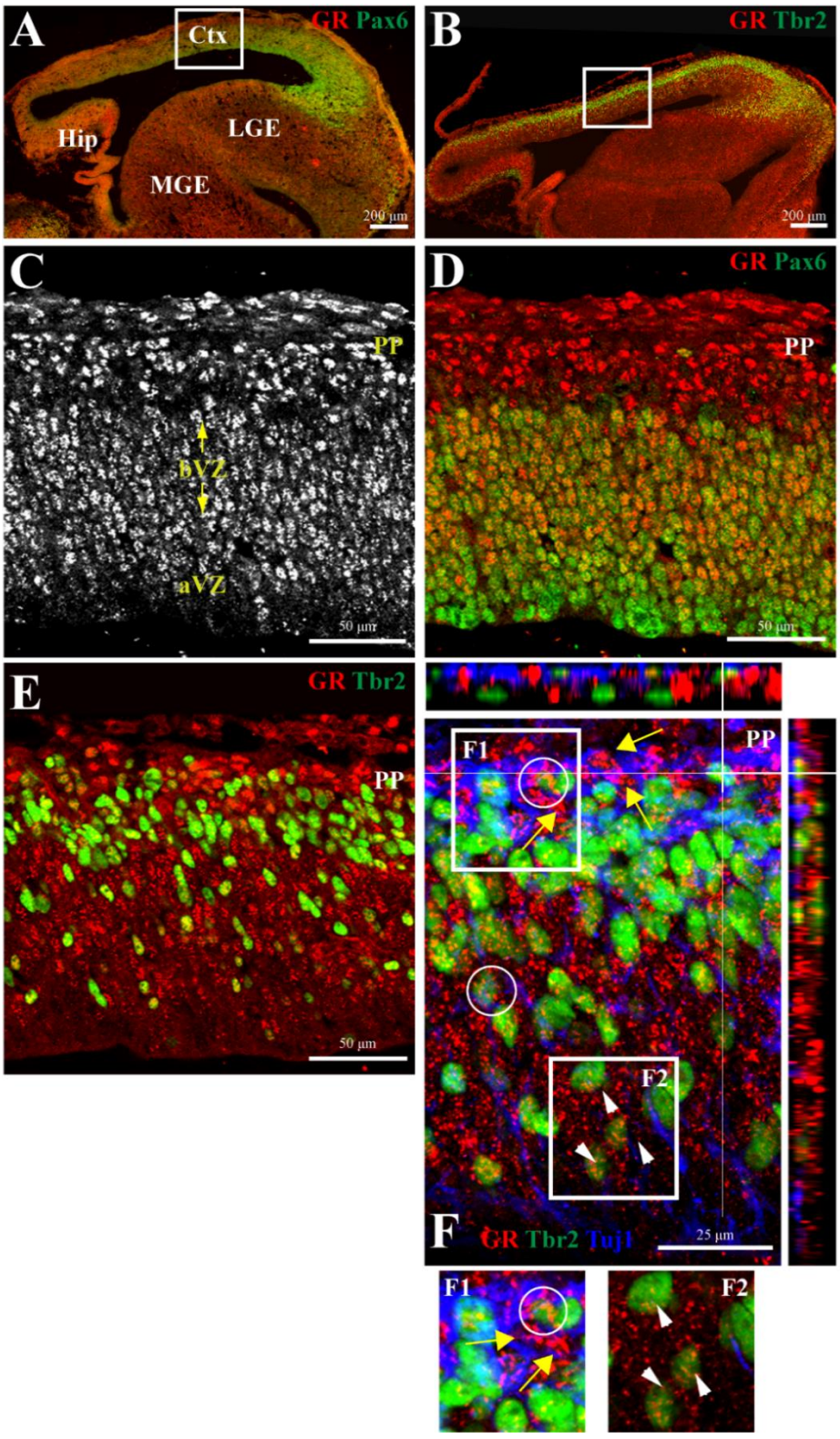


Figure 11. GR is expressed in the nucleus of Radial Glia Cells (RGCs), Intermediate Progenitors (IPCs) and early neurons of the E11.5 dorsal telencephalon.

Sagittal sections throughout the telencephalon of E11.5 mouse embryos stained with BuGR2 (red) and the RGC-marker, Pax6 (A, green), or the IPC-marker, Tbr2 (B, green). GR colocalizes with Pax6- (A, orange/yellow) and Tbr2-positive cells (B, orange/yellow), throughout the rostrocaudal extent of the telencephalon. (C, D). Single optical section of the area boxed in panel A (similarly, the boxed area in panel B is shown in E). Panel C, shows GR expression in grayscale, whereas, panel D shows the same section with BuGR2 and Pax6 staining. (E). In IPCs, GR colocalizes with Tbr2 (seen as yellow/orange) in the nucleus. (F). Confocal z-stack, of triple immunostaining with neuronal marker Tuj1 (blue), Tbr2 and BuGR2, shows expression of GR in early neurons of the E11.5 cortex (yellow arrows, inset F1) and in nascent neurons derived from IPCs (circles, Tuj1 (blue)/Tbr2/GR (yellow)-positive cells). Example orthogonal projections show GR positive cells with colocalization of Tbr2 and Tuj1 or Tbr2 only. White arrows indicate GR expression in IPCs (yellow, Tbr2 only, inset F2). Boxed areas F1 and F2 are shown in higher magnification at the bottom of panel F. C-E, single optical sections. Ctx, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence.

2.3.2.3 Differential Subcellular Localization of GR in E13.5 NSPCs

During mid-neurogenesis (E13.5-E14.5) the IPC population reaches its peak size and forms the subventricular zone (SVZ). The upper boundary of the SVZ is identified by the expression of Tbr2 (Figs. 12A, B). In addition, during mid-neurogenesis, neurogenic divisions from both, RGCs and IPCs generate stellate and pyramidal neurons, which populate the nascent cortical plate (Stancik et al., 2010). Double IIF for BuGR2 and Pax6 or Tbr2 on sections of E13.5 telencephalons revealed that GR is expressed in both progenitor populations (Figs. 12B, C). Contrary to the small number (single layer) of RGCs expressing predominantly cytoplasmic GR at E11.5, GR was localized mainly within the cytoplasm of the majority of RGCs in the aVZ at E13.5, spanning a zone of approximately 3 cell diameters (Figs. 12B, C and 13I, J, arrows). Thus, the number of RGCs in the aVZ expressing GR in their cytoplasm increased on E13.5, compared to E11.5. Similar to what we observed on E11.5, RGCs in the E13.5 bVZ predominantly expressed nuclear GR (Figs. 12B-D, 5E and Sup. Figs. 2A-C), while in Tbr2-positive cells GR was nuclear (Figs. 12B, D). Triple IIF for BuGR2, Tuj1 and Tbr2 showed that GR is nuclear both in mature neurons of the cortical plate (Tuj1-positive/Tbr2-negative, Fig. 4D-G, arrows) and in nascent neurons (Tuj1-positive/Tbr2-positive, Fig. 12D, circles and Appendix A Figs. 36D-F). In Figure 5 we provide a comparison between the subcellular localization of the GR protein within NSPCs of the E11.5 telencephalic neuroepithelium (Figs. 13A-D) and in NSPCs of the E13.5 neuroepithelium (Figs. 13E-K). Note the distinct cytoplasmic localization of GR in NSPCs of the E13.5 aVZ (Figs. 13H, J) which extends approximately 3 cell diameters beyond the aVZ, compared to a less prominent cytoplasmic GR profile in E11.5 NSPCs of the aVZ (Figs. 13A, D).

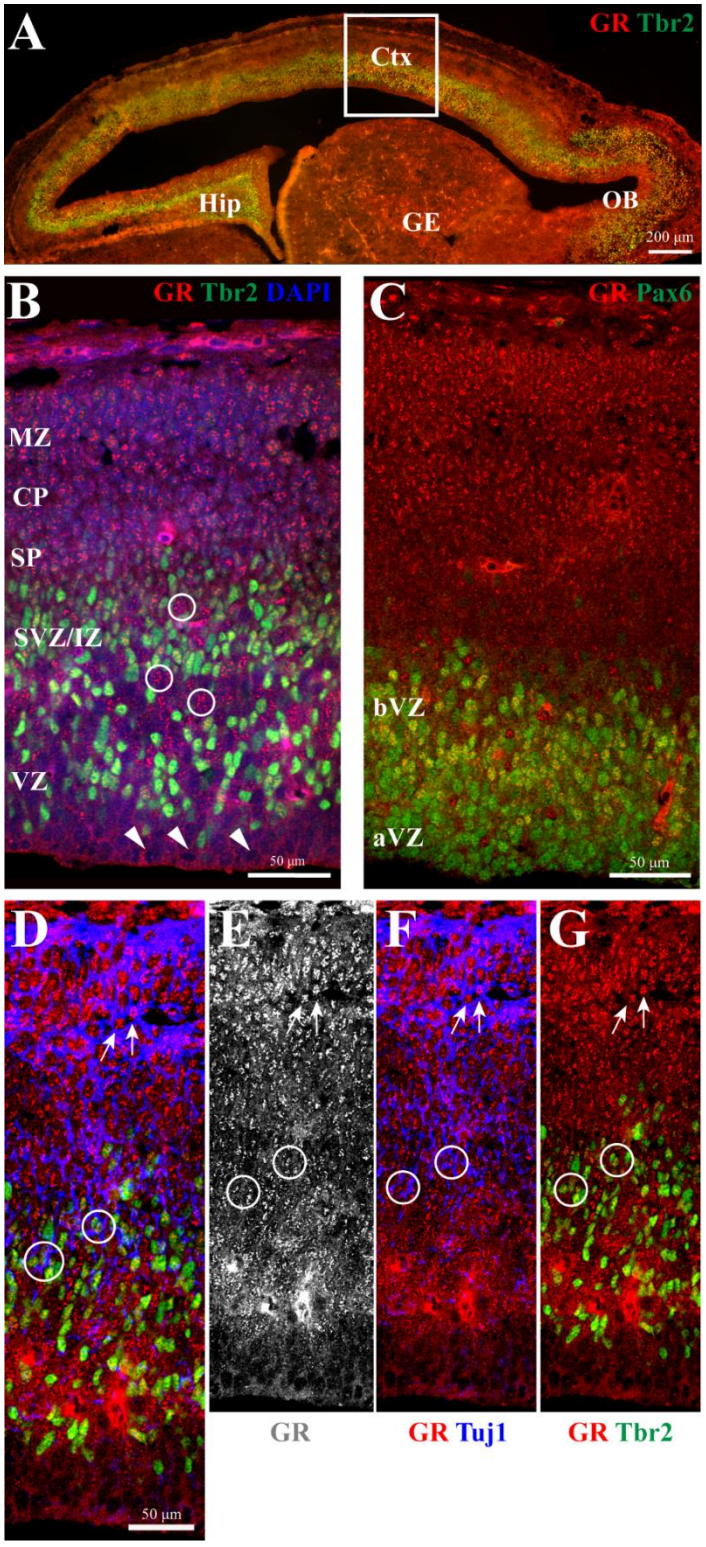

A
GR Tbr2
Ctx
Hip
GE
OB
200 µm
B
GR Tbr2 DAPI
MZ
CP
SP
SVZ/IZ
VZ
50 µm
C
GR Pax6
bVZ
aVZ
50 µm
D
50 µm
E
F
G
GR
GR Tuj1
GR Tbr2

Figure 12. GR is expressed in the nucleus of RGCs and IPCs of the E13.5 dorsal telencephalon.

(A-C).Sagittal section through the telencephalon of an E13.5 mouse embryo stained with BuGR2 (red) and Tbr2 (green) (B), or BuGR2 (red) and Pax6 (green) (C). The inset in A, shows the relative anatomical position of images in B and C. The immunostaining reveals that GR is expressed in E13.5 IPCs (B), RGCs (C), in mature neurons (D-F, Tuj1-positive/Tbr2-negative, arrows) and in nascent neurons derived from IPCs (D-G, Tbr2-positive/Tuj1-positive, circles). In the aVZ, GR is primarily cytoplasmic (B, arrowheads), whereas in the bVZ/SVZ is primarily nuclear (B, circles). Images B-D, single optical sections. Hip, hippocampus, GE, ganglionic eminence, Ctx, cortex, OB; olfactory bulb; MZ, mantle zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

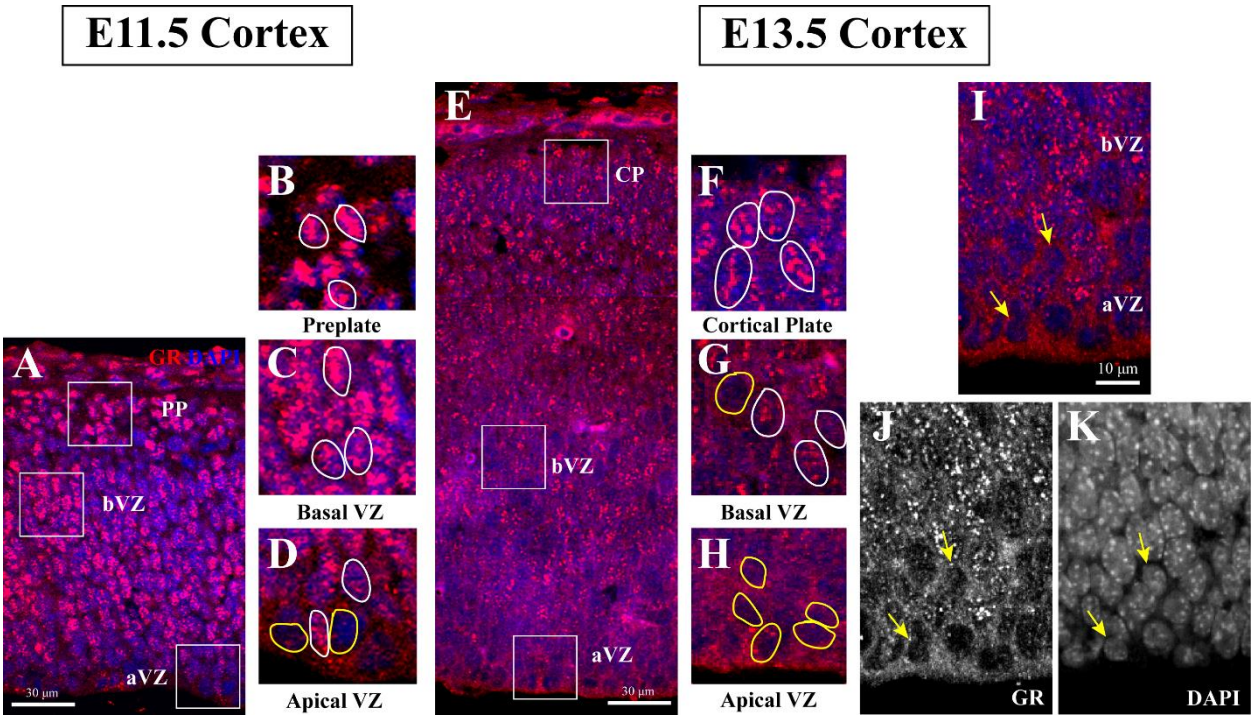


Figure 13. Differential subcellular distribution of GR in NSPCs of the E11.5 VZ compared to its subcellular profile in NSPCs of the E13.5 VZ.

(A, E) Sagittal sections from an E11.5 and an E13.5 dorsal telencephalon, respectively, stained for BuGR2 (red) and counterstained for DAPI. Insets in A and E, show the position of panels B-D and F-H, respectively. (A-D) In the E11.5 NSPCs GR is mainly nuclear with the exception of a layer of NSPCs in the aVZ (panel D, yellow outlines). (E-H) In the E13.5 aVZ, the number of NSPCs expressing cytoplasmic GR is increased, forming a zone which extends up to 3 cell nuclei diameters in the aVZ (E, H), whereas, NSPCs of the E13.5 basal VZ (bVZ), contain both cytoplasmic (G, yellow outlines) and nuclear GR (G, white outlines). Outlines indicate individual cell profiles. Cells in the cortical plate (F), express relatively high nuclear GR. (I-K) High magnification of the E13.5 VZ, where the cytoplasmic GR expression in the aVZ is seen in more detail (arrows point to individual cell/nuclei profiles). Note also the high nuclear GR content in neighbouring cells that extents into the bVZ. Single optical sections, inset size 30 µm.

2.3.2.4 The Expression of GR is Highest in the Hippocampus and Caudal Cortical Regions both at E11.5 and E13.5.

Neurogenesis in the cortex proceeds in a rostral-high caudal-low gradient (Shimada and Langman, 1970). Accordingly, neurons in rostral areas are generated earlier than those of caudal areas. Moreover, gradients of morphogenetic and transcription factors are important for development, since they are involved in setting borders of brain areas (Caviness et al., 2009; Kwan et al., 2012; Rakic, 1988). GR expression appeared to differ along the rostro-caudal axis of the developing dorsal telencephalon. This was most apparent when GR expression was visualized using a heatmap generated by NIH ImageJ software (Fig. 14) (Schneider et al., 2012). Specifically, in low magnification images both at E11.5 (Figs.14A-C) and E13.5 (Figs. 14H, I), GR levels were highest in the hippocampal neuroepithelium. In addition the caudal cortical neuroepithelium adjunct to the hippocampus expressed relatively higher GR levels in comparison to more rostral cortical areas. Side to side comparison of GR expression in high magnification confocal images of the rostral and perihippocampal neuroepithelium at E11.5 (Figs. 14C-G) revealed a smaller number of NSPCs expressing very high GR levels (red) in the rostral area (Fig. 14D) compared to the perihippocampal cortical area (Fig. 14E) and the hippocampus proper (Fig. 14F). The high GR expression in NSPCs and differentiating cells of the hippocampus compared to the cortex, is also seen in a coronal section of the E11.5 telencephalon (Fig. 14G). Also, GR levels were very high in neurons of the preplate (Figs. 14D, E, G, magenta outlines; red cells). Similarly in high magnification confocal images of the E13.5 telencephalon (Figs. 14J-L), we observed relatively higher GR expression (number of red colored cells) in the hippocampal neuroepithelium (Fig. 14L) compared to rostral cortical neuroepithelium (Fig. 14J). Moreover, neurons in the cortical plate, expressed high levels of GR

compared to NSPCs (Fig. 14J, magenta outline). Thus, the highest expression of GR was observed in NSPCs and differentiating cells of the hippocampal area at E11.5 and E13.5.

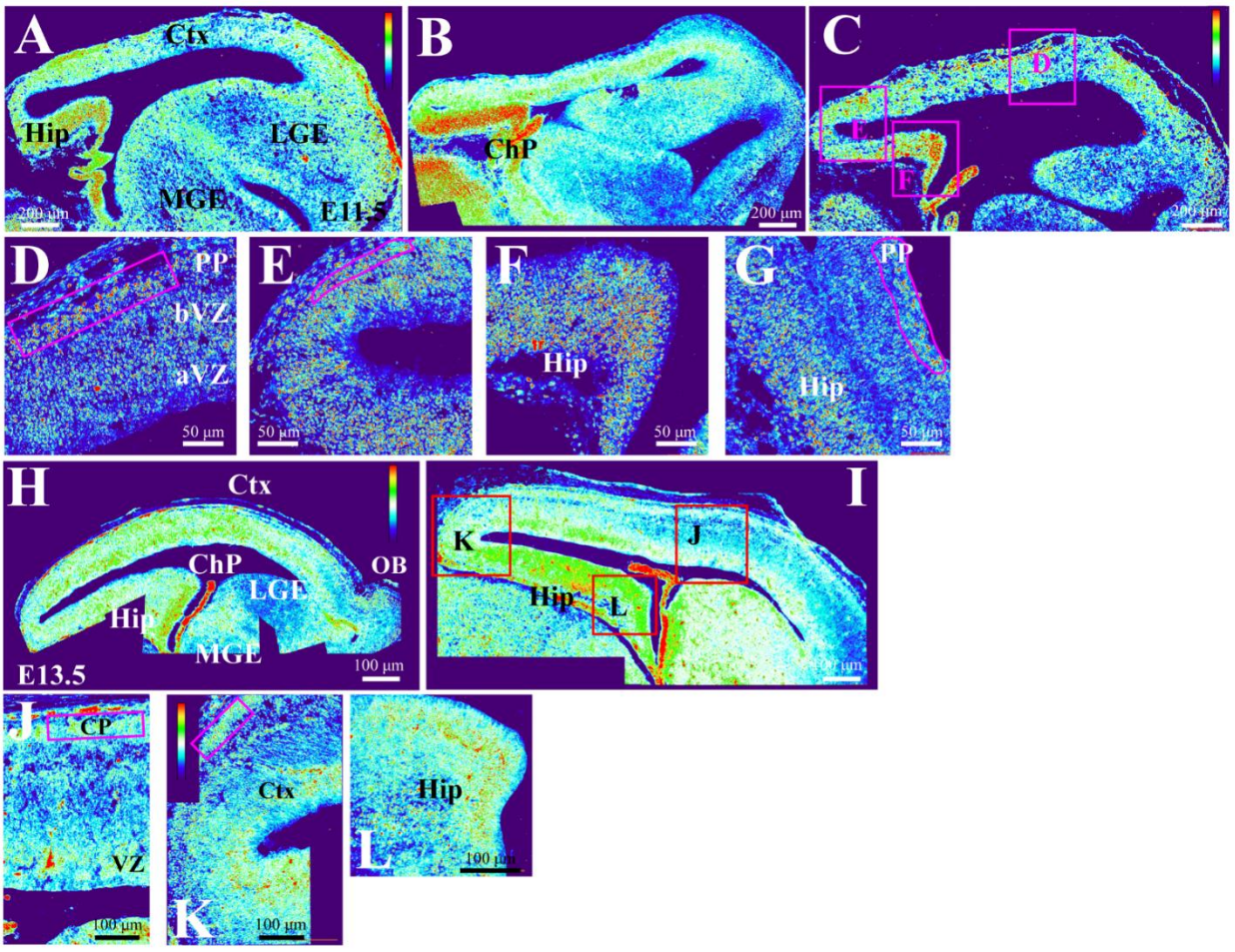


Figure 14. GR expression follows a caudal to rostral gradient in the developing telencephalon.

Sagittal sections from E11.5 (A-G) and E13.5 telencephali (H-L), in which GR expression is visualized as a heatmap generated by NIH ImageJ software (Schneider et al., 2012). The hippocampus (Hip) and the adjunct cortical neuroepithelium, express the highest levels of GR both on E11.5 (F, G) and on E13.5 (L). (D-G) Confocal z-stacks of a sagittal (D-F) and of a coronal (G) section through the telencephalon on E11.5 (C-G) and of a sagittal section on E13.5 (J-L). Insets in C, indicate the anatomical position of panels D-F. Preplate (PP) and cortical plate (CP) neurons express high GR levels (D, E, G and J, K respectively; magenta outlines). The insets in panels J and K, indicate the anatomical position of confocal images J-L. Also, note that the chorioid plexus (ChP) is another area with very high GR levels at both ages (E11.5: A-C; E13:5 H, I). Images C-G, J-L, confocal z-stacks. Ctx, cortex; LGE/MGE, lateral and medial ganglionic eminence; aVZ/bVZ, apical and basal ventricular zone.

2.3.2.5 In the Perinatal Dorsal Telencephalon, GR is Nuclear, Restricted to Specific Cortical Layers and Present in Late RGCs and IPCs.

NSPCs switch from making neurons to glia at late embryonic developmental stages (Kriegstein and Alvarez-Buylla, 2009). Therefore to determine if GR protein is also found in glia or gliogenic progenitors, we examined its expression during late stages of fetal development i.e., E17.5. GR was expressed throughout the dorsal telencephalon (Fig. 15A). In addition, on E17.5 GR was expressed in the cytoplasm and the nucleus of Pax6-positive RGCs residing at either the ventricular or the abventricular surface (Fig. 15C-E). Similar to earlier ages, GR was primarily nuclear in IPCs on E17.5 (Figs.15F-H). Moreover we observed that GR is prominently expressed in blood vessels (Fig. 15C-E, stars). In the cortical plate at E17.5, GR was expressed at higher levels in distinct neuronal populations, i.e. in putative layers IV/V and the subplate (Fig. 15B). To verify that the GR-positive cells in the cortical plate are indeed layer V neurons, we performed double IIF for BuGR2 and a layer V-specific marker, Ctip2, at P0.5 when layer V is distinct. These results revealed that the majority of Ctip2-positive cells expressed nuclear GR. Moreover, the relatively high levels of GR in the nucleus of layer V cells (Figs. 15I-L) were observed at all rostral to caudal levels.

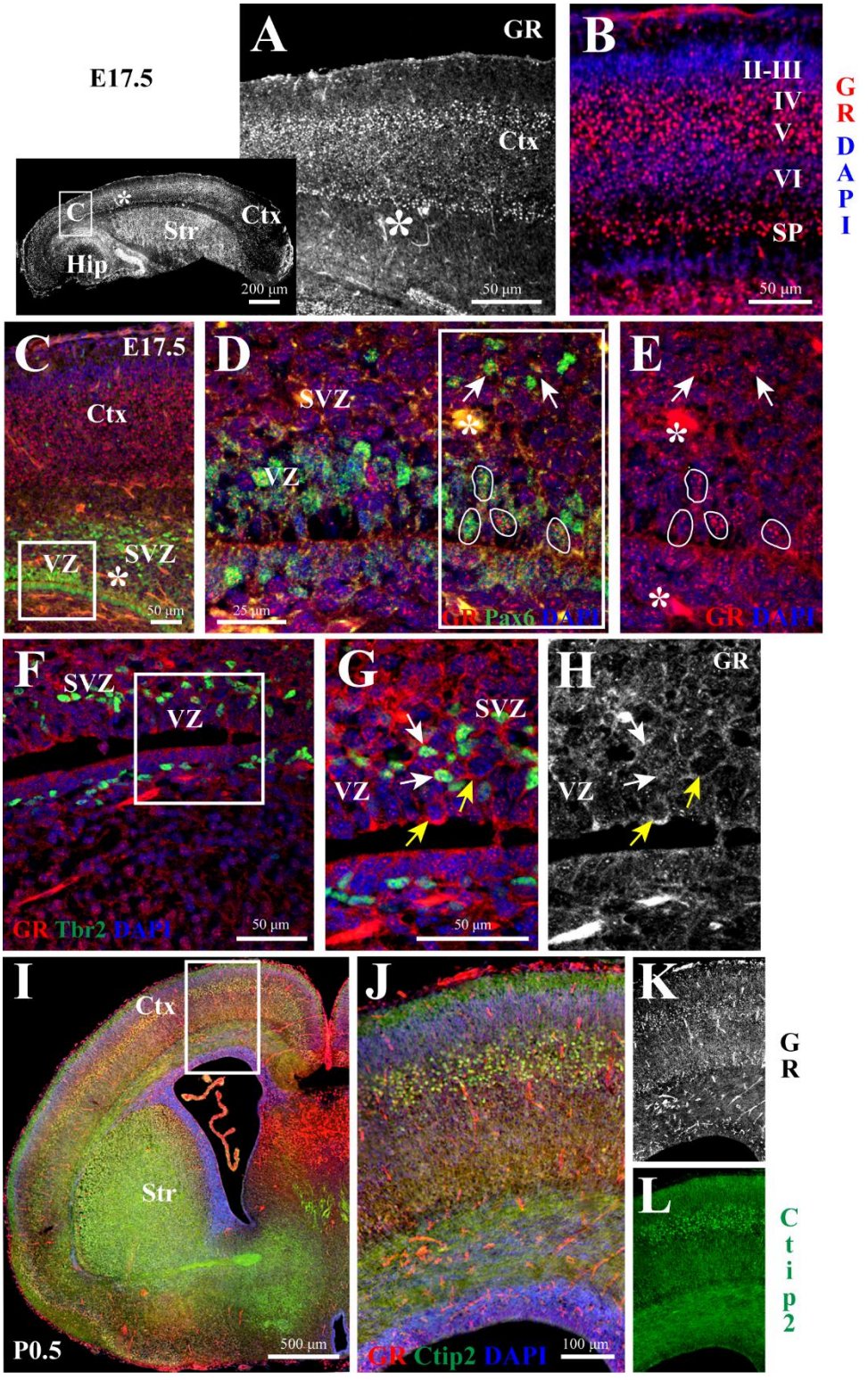


Figure 15. Expression of the GR in the E17.5 cortex.

(A) E17.5 horizontal section through the telencephalon, stained for BuGR2 (grayscale). In the inset, the anatomical position of panels A and B is indicated by the star (*), while box C indicates the anatomical position of panel C. (B) Nuclear expression of GR (red) in cells of putative layers IV/V, VI and in the subplate (SP). (C-E) Horizontal section through the cortex stained for BuGR2 (red), Pax6 (green) and counterstained with DAPI. The box in panel C, is shown in higher magnification in D, whereas the boxed region in D is shown in E with only BuGR2 and DAPI. (D, E) Outlines in panel D and E indicate individual cell profiles, with colocalization of Pax6 and GR (orange/yellow) in RGCs. GR expression in blood vessels is indicated by asterisks (*). (F-H) Horizontal section taken from the same anatomical level as D, stained with BuGR2 (red), Tbr2 (green) and DAPI (blue). The boxed area represents the images in G and H. Expression of GR in IPCs cells is indicated by colocalization of GR (red) and Tbr2 (green) (G, yellow nuclei, white arrows), whereas cytoplasmic GR is also evident in the VZ (G, H, yellow arrows). (J, K) Coronal section of the telencephalon at P0.5. GR expression in layer V neurons as seen by overlap of layer V marker, Ctip2 (green), and BuGR2 (red) (yellow nuclei). C-H, single optical sections. Ctx, cortex. Hip, hippocampus, Str, striatum.

2.3.3 Expression of GR in the Developing Ventral Telencephalon.

The glutamatergic cells of the dorsal telencephalon predominantly arise from Pax6- and Tbr2-expressing NSPCs of the dorsal telencephalic neuroepithelium. In contrast, GABA-ergic interneurons are derived from NSPCs in the ventral telencephalon, which consists of the medial, lateral and caudal ganglionic eminences (MGE, LGE and CGE respectively). We therefore examined the expression of GR protein in NSPCs of the GEs. At E11.5, similar to the dorsal telencephalon, GR was expressed in NSPCs of the GEs (Figs. 16A, B) and as shown by colocalization of GR with the progenitor marker Sox2 (Fig. 16C). GR was also expressed in NSPCs in the corticostriatal boundary (Figs. 16A, B star), an area important for cell migration and patterning (Assimacopoulos et al., 2003). In addition, in NSPCs of the GE progenitor zone, we observed a mixed pattern of subcellular GR localization. Accordingly, some cells had relatively high levels of nuclear GR, while others expressed mainly cytoplasmic GR with minimal nuclear staining (Figs. 16A, C). Similarly, in differentiating neurons of the GE we observed both nuclear and cytoplasmic GR expression (Figs. 16A, circle). This pattern was preserved at all developmental ages examined. In general, NSPCs located on the aVZ surface primarily expressed GR in their cytoplasm (Figs. 16A, C insert; arrows pointing to VZ). In contrast abventricular NSPCs and differentiated cells primarily expressed GR in their nucleus (Figs. 16D, E). To verify that GR is expressed in differentiated cells we used the interneuron marker calbindin (CB), and we observed nuclear GR expression in CB-positive cells (Figs. 16D, E arrows). On E13.5, in Sox2-positive NSPCs cytoplasmic GR expression was more pronounced in the aVZ. (Figs. 16G,-J), Moreover, GR was also expressed in the nucleus of GABA-ergic cells of the E13.5 GEs (Figs. 16K, L).

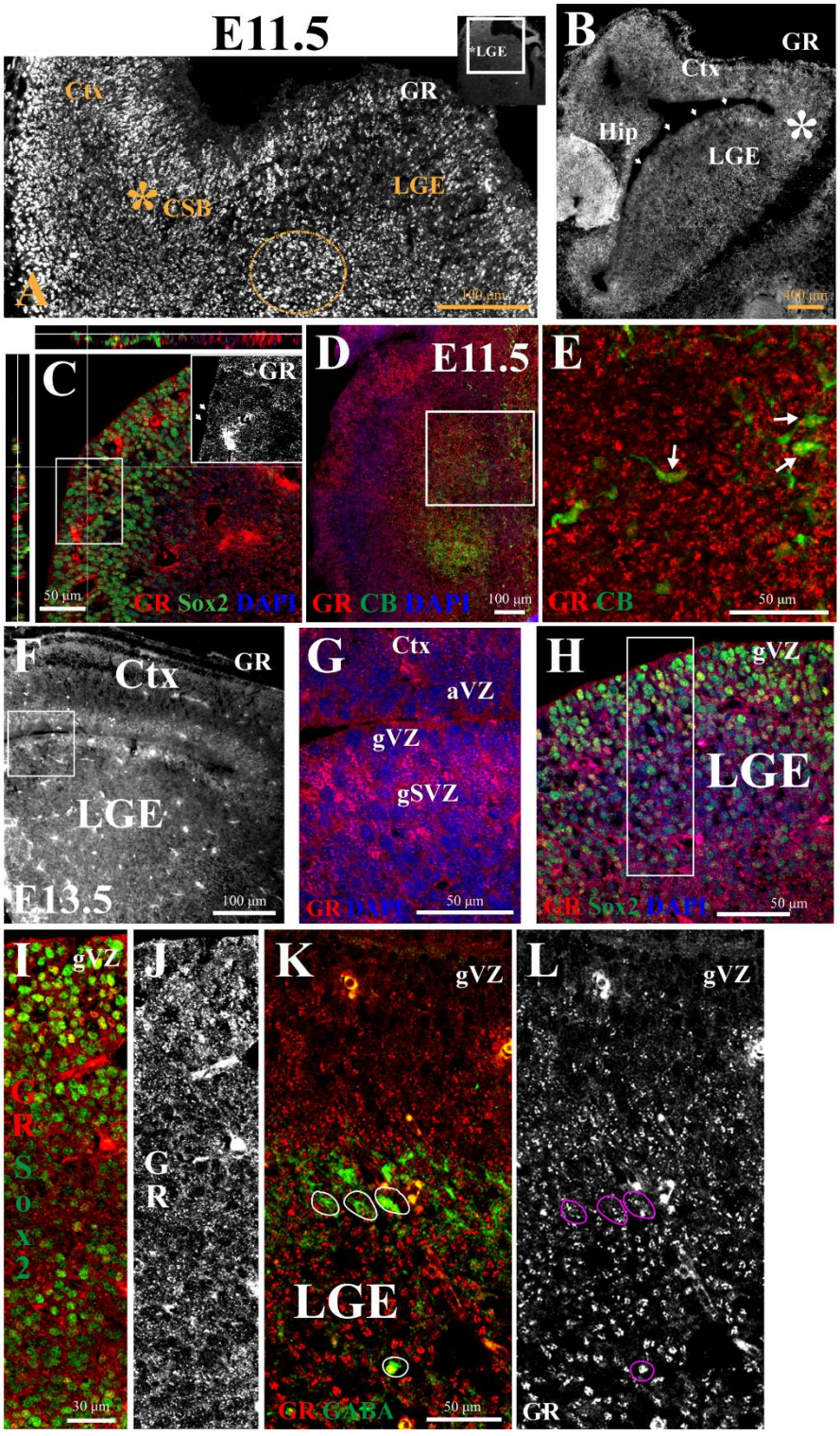


Figure 16. GR is expressed in NSPCs and differentiated cells of the E11.5 and E13.5 Ganglionic Eminences.

(A-E) E11.5 and (F-L) E13.5 ganglionic eminences. (A) Single optical section of an E11.5 coronal section through the telencephalon, stained with BuGR2 (grayscale). GR is expressed in the lateral ganglionic eminence (LGE), in NSPCs of the corticostriatal boundary (CSB, *) and in differentiating cells (circle). The boxed region in the inset shows the anatomical position of panel A. (B) Sagittal section of the telencephalon on E11.5. GR is expressed throughout the LGE, but in lower levels in cells of the apical surface (arrows). (C-E) Sagittal sections through the ganglionic eminence (GE), approximately at the level of A. (C) GR is expressed in NSPCs of the GE as seen by colocalization of the NSPC marker, Sox2 (green) and of BuGR2 (red). Cells on the apical surface primarily express cytoplasmic GR, as also seen in the inset of the boxed area. The arrows point to individual cell profiles with cytoplasmic GR expression (grayscale). Example orthogonal projections show GR positive cells that also express Sox2. (D-E) GR is also expressed in the nucleus of interneurons as seen by colocalization of calbindin (CB, green) and BuGR2 (red). The boxed area in D indicates the anatomical position of image E. Arrows in E indicate individual cell profiles coexpressing CB and GR. (F) Sagittal section of an E13.5 telencephalon containing the LGE, stained for BuGR2 (grayscale). Panel (G) is a single optical section of the boxed area in F, showing the LGE and part of the dorsal telencephalon, at higher resolution. GR expression in NSPCs of the GE has a mixed nuclear and cytoplasmic profile, but apical NSPCs primarily express cytoplasmic GR, similar to NSPCs in the aVZ of the cortex. (H-L) Single optical sections through the E13.5 LGE. (H-J) GR is expressed in NSPCs as shown by colocalization (yellow) with the NSPC marker Sox2. The boxed region in H is shown in higher magnification in I-J. (K-L) Also, GR is expressed in GABA-ergic interneurons, as seen by colocalization of GABA (green) and GR (red). Outlines show individual cell profiles. A, C, G-L, single optical sections; D, E, z-stacks. Hip, hippocampus; Ctx, cortex; aVZ, apical ventricular zone; bVZ, basal VZ; gVZ, ganglionic VZ; gSVZ, ganglionic subventricular zone.

2.3.4 GR is Highly Expressed in the Hippocampus From Early Developmental Stages.

GCs play a dominant role in learning and memory processes mediated by the hippocampus, both in the neonate and the adult brain. In addition, GCs and stress reduce adult hippocampal neurogenesis (Gould et al., 1997). Therefore, to evaluate the pattern of GR localization in embryonic NSPCs of the developing hippocampus, we examined telencephalic sections through the hippocampus from E11.5 embryos. In NSPCs of the E11.5 hippocampus, GR was expressed in relatively high levels (Figs. 11B, 17A and B) and in comparison to the rest of the telencephalon, the hippocampus had the highest expression levels of GR (Figs. 14C, D, G). Interestingly, the relatively high expression of GR extended beyond the Pax6 boundary (Figs. 17A, B). Similar to the cortex at this age, GR was expressed mainly in the cytoplasm of hippocampal NSPCs located in the apical hippocampal VZ, whereas cells located in the SVZ and the differentiating zone of the hippocampus, primarily expressed nuclear GR (Fig. 17B). In addition, GR was present in Tuj1-positive cells, putatively representing early hippocampal pyramidal neurons (Fig. 17C, arrows) and in nascent neurons expressing both Tuj1 and Tbr2 (Fig. 17C, circles).

As noted for the E13.5 cortical neuroepithelium, the number of NSPCs in the apical hippocampal VZ expressing GR primarily localized in the cytoplasm was increased compared to E11.5 (Fig. 17D, E arrowheads). In contrast, NSPCs of the hippocampal SVZ, the inner hippocampal mantle and putative neurons of the subiculum expressed relatively high levels of GR in their nuclei (Figs. 17D, arrows indicate differentiated cells, E, star indicates SVZ area).

At perinatal stages, i.e., E16.5-E17.5, hippocampal NSPCs mainly give rise to CA1 pyramidal cells, while concurrently postmitotic cells are migrating towards their respective laminas in the hippocampus (Soriano et al., 1989a; Soriano et al., 1989b). In E17.5 Pax6-expressing

progenitors (Duan et al., 2012), GR was present both in the cytoplasm and nucleus (Figs. 17G-J, outlines in I) similar to the cortex, whereas in cells of the subiculum, areas CA1-3 of the Cornus Ammonis and the developing DG (Figs. 17G-J), GR was predominantly nuclear. The circled area in Figure 17G, delineates the DG which contained GR-positive nuclei (red). Figure 17J shows CA1 pyramidal cells that contained relatively high levels of nuclear GR (outlines).

2.3.5 GR is Highly Expressed in NSPCs Throughout OB Morphogenesis and Delineates Specific Cell Layers in the Perinatal OB.

The formation of OB initiates on E9.5 from cells in the surface neuroepithelium. However, neurons destined for the mature OB begin to migrate from the telencephalon around E13.5, (Hinds, 1968; Hinds, 1972), a stage where the OB is clearly distinguished as a bud at the rostral end of the embryonic telencephalon. Therefore, we examined GR protein expression in Pax6- and Tbr2-expressing NSPCs of the embryonic OB from E13.5. At this age, GR showed a predominant cytoplasmic localization in Pax6-positive NSPCs with a minimal nuclear localization in the developing OB (Fig. 18A). Similar to cortical IPCs, Tbr2-positive IPCs in the OB expressed nuclear GR (Figs.18B). The strongest expression of GR at this age was observed in differentiating neurons, which contained GR in their nuclei (Figs. 18A, B). This pattern of expression persisted until E17.5 where the most prominent expression was observed in the nucleus of mitral cells (Figs. 18C, D arrows). In general, the expression of GR in the olfactory bulb neuroepithelium, followed the same pattern we had observed for the cortical, ganglionic eminences and hippocampal neuroepithelium. Specifically, on E11.5, GR expression in the VZ neuroepithelium is mixed, with a small number of cells in the aVZ expressing cytoplasmic GR, while others had low levels of GR in their nucleus. In the bVZ and upper layers, GR was

predominantly nuclear and was expressed in relatively high levels, while on E13.5 the number of NSPCs in the aVZ expressing GR in their cytoplasm was increased.

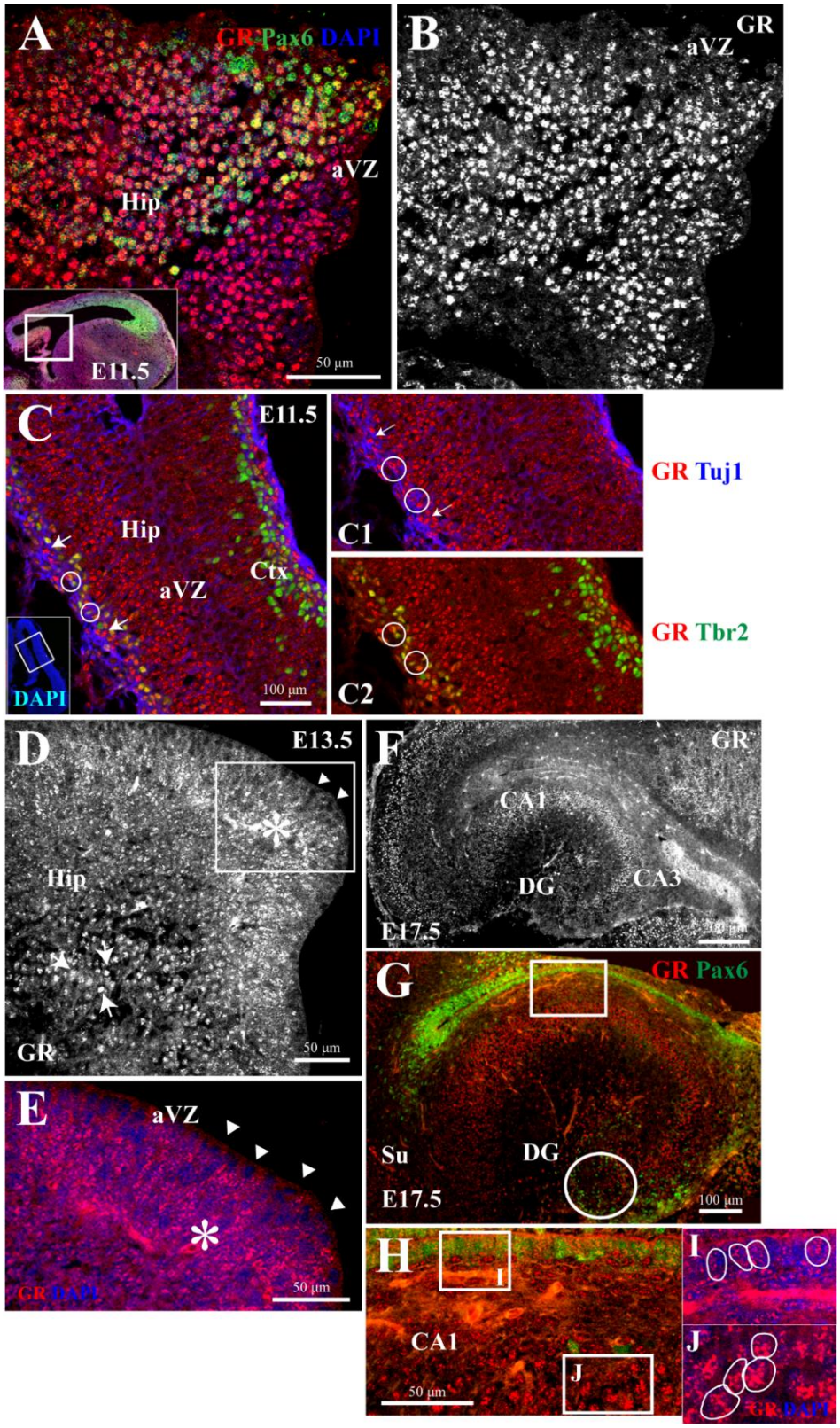


Figure 17. GR expression in NSPCs of the developing hippocampus.

(A) E11.5 sagittal section of the hippocampus stained for Pax6 (green), BuGR2 (red) and counterstained for DAPI. The boxed area in the inset indicates the position for panels A, B. Panel B shows GR expression in grayscale.(C) E11.5 coronal section through the hippocampus immunostained for Tuj1, Tbr2 and BuGR2 (single optical section). The boxed area in the inset represents the area shown in panel C. GR is present in NSPCs (A-C) and early neurons (C, arrows) in the hippocampus at E11.5. NSPCs on the apical surface primarily express cytoplasmic GR while abventricular cells (A-C) primarily express nuclear GR. Circles in C indicate nascent neurons (Tbr2-positive/Tuj1-positive). **(**D) Single optical section of a E13.5 sagittal section stained for BuGR2 (grayscale). GR is expressed in NSPCs on the apical surface of the hippocampus (arrowheads) and in differentiating cells of the hippocampal anlagen (arrows). Panel E is a magnification of the boxed region in D, showing a primarily cytoplasmic GR expression in NSPCs on the apical surface of the hippocampus (arrowheads), whereas abventricular NSPCs primarily express nuclear GR (* star). (F) GR expression (BuGR2, grayscale) in the E17.5 hippocampus (horizontal section). GR is expressed in areas CA1 and CA3. (G-J) Single optical sections of the hippocampus. Pax6-positive NSPCs lining the apical surface of the hippocampus express GR (boxed region also shown in higher magnification in H). (H) GR staining in Pax6-positive cells lining the top of the CA1 (outlined in I) and in pyramidal cells of the CA1 (outlined in J). The newly-forming DG also expresses GR (circle, red nuclei). Ctx, cortex; Str, striatum; Hip, hippocampus; Su, subiculum; CA, Cornus Ammonis.

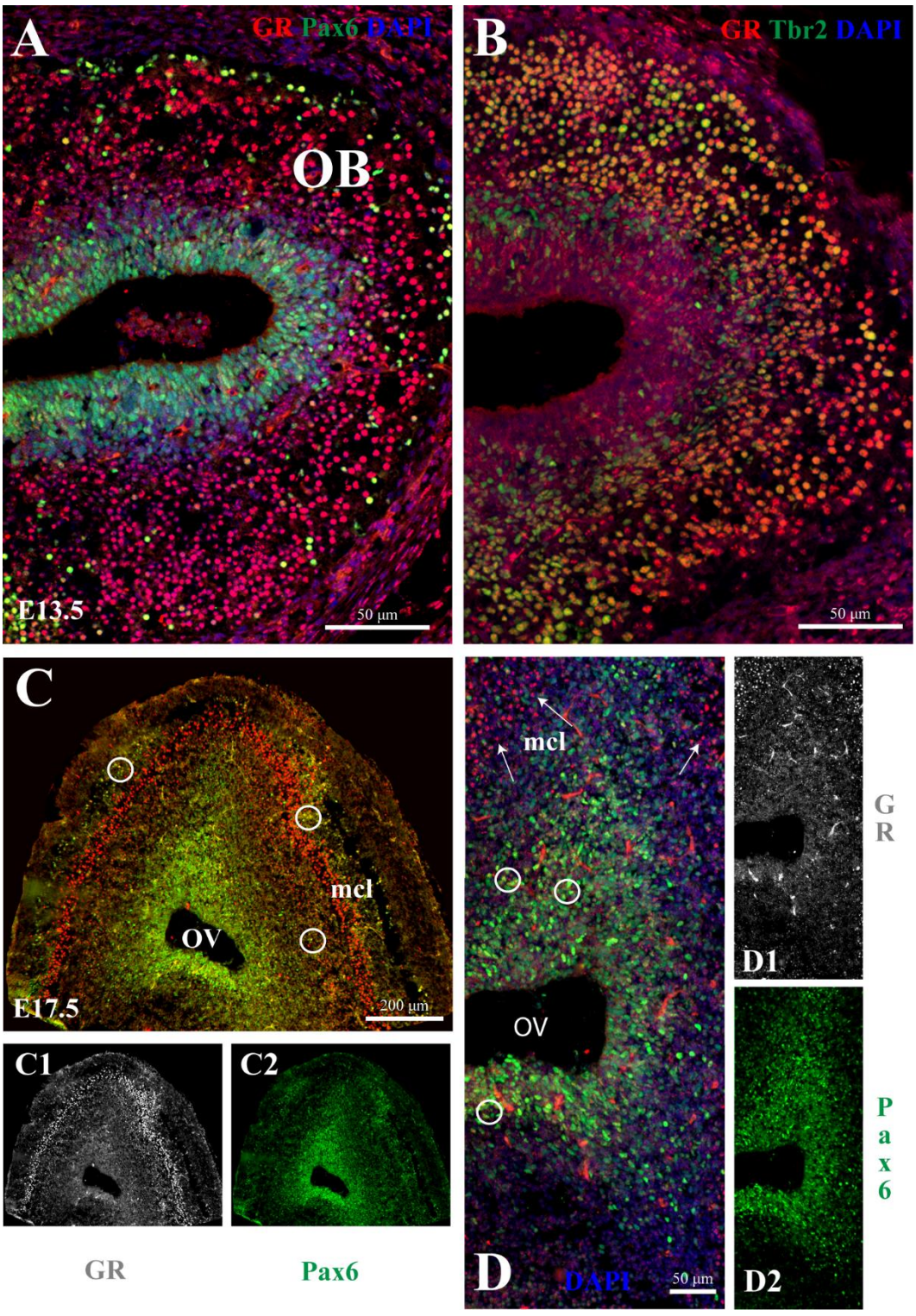


Figure 18. Expression of GR in NSPCs the developing Olfactory bulb at E13.5 and E17.5

(A) Sagittal section of an E13.5 telencephalon showing expression of GR (red) in Pax6-positive radial glial cells. Colocalization of GR and Pax6 is indicated by the yellow color of the cell nuclei. (B) Colocalization of GR with Tbr2-positive cells (yellow) in the developing olfactory bulb (OB) at E13.5. (C, D) Immunostaining for Pax6 (green) and BuGR2 (red) on a horizontal section of the E17.5 OB. Panel D is a single optical section of the OB showing localization of GR in Pax6-positive progenitors of the OB, mainly in abventricular positions (yellow nuclei). GR staining is also present in nuclei of the putative mitral cell layer (mcl, arrows in D), as well as in Pax6-positive cells of the ventricular surface and the outer layer of the OB (yellow, circles). A, B, D: single optical sections. OV, olfactory bulb ventricle.

2.4 DISCUSSION

In this study we demonstrated that GR protein is expressed from E11.5 in RGCs, IPCs and neurons of the telencephalic neuroepithelium and is predominantly nuclear within the radial extent of the neuroepithelium. Specifically, in the dorsal telencephalon GR was expressed in Pax6-positive RGCs, Tbr2-positive IPCs, and in migrating and pioneer neurons of the preplate. In a small number of RGCs lining the apical VZ, GR had a predominantly cytoplasmic localization, whereas in the basal VZ and the preplate, GR was nuclear. Moreover, nuclear expression levels increased in an apical to basal pattern. However, in the E13.5 neuroepithelium the population of NSPCs in the aVZ primarily expressing cytoplasmic GR was increased, whereas in cells of more basal layers i.e., SVZ NSPCs and neurons, GR gradually progressed from a mixed cytoplasmic and nuclear localization, to a predominant nuclear localization in differentiating neurons. This neurodevelopmental stage-specific shift from nuclear to cytoplasmic localization was also observed in the ganglionic eminences, hippocampus and olfactory bulb. Moreover, this differential subcellular profile of GR in NSPCs was maintained until perinatal stages i.e., E17.5.

2.4.1 *Nuclear GR in the Absence of GC-Ligand?*

Given that GR has the capacity to shuttle between the nucleus and cytoplasm (Madan and DeFranco, 1993), the steady state localization of the receptor is determined by rate-limited transport. In tissues of intact animals, many factors could potentially influence the ability of physiological levels of GC to drive nuclear import of GR (Vandevyver et al., 2011). Albeit, nuclear GR localization in the absence of hormone ligand was observed in cortical pyramidal

cells of 3-month old adrenalectomized rats by Pekki et al., (1992). Similarly, we observed nuclear GR in NSPCs of the E11.5 neuroepithelium (Fig. 11), a stage where endogenous GCs are expected to be minimal. The endogenous production of GCs in the mouse embryo is established around E16 in order to promote organ maturation and preparation for the upcoming birth (Venihaki et al., 2000). In parallel, at E16, the hypothalamus-pituitary-adrenal axis feedback loop becomes active (Reichardt and Schutz, 1996). Therefore, our results suggest that unliganded GR may be responsive to region-specific signaling pathways that drive transport into and stable accumulation of GR within the nucleus at early neurodevelopmental stages.

2.4.2 *GR Expression in NSPCs and Subcellular Expression*

Our results show that expression of GR within the radial extent of the neuroepithelium changes with development. Specifically, on E11.5 a small number of RGCs in the aVZ primarily expressed cytoplasmic GR, whereas the rest of the NSPCs expressed low levels of nuclear GR in apical areas but higher levels in basal areas. On E13.5, the number of RGCs primarily expressing cytoplasmic GR was increased in the dorsal cortex and throughout the telencephalon from E11.5 to E17.5. Nevertheless, GR remained nuclear in IPCs irrespective of their position in the neuroepithelium. Therefore, cytoplasmic localization of GR appears to be specific for embryonic RGCs and NSPCs adjacent to the ventricle. It is possible that specific growth factors present in the cerebral spinal fluid limit GR nuclear localization.

In the adult hippocampus, GR colocalizes with the stem cell protein nestin, both *in vivo* and *in vitro* (Garza et al., 2011; Reimer et al., 2009). Nestin has a regulatory role on the function of GR, and specifically a nestin/vimentin network maintains the unliganded GR in the cytoplasm and is associated with an increased proliferative capacity. Since Pax6-positive radial glia in the aVZ

express high levels of nestin in contrast to their IPC progeny in the bVZ/SVZ, from which only a minimal proportion expresses nestin on E14.5 (Dahlstrand et al., 1995; Englund et al., 2005), it is tempting to speculate that nestin may also have a similar role in the subcellular GR localization we observed. Interestingly, Fitzsimons et al. (2008) showed that in adult hippocampal NSPCs, nuclear translocation of GR is controlled by cytoskeletal components, making this a plausible mechanism operating in the embryonic NSPC niche.

2.4.3 *A Role for Unliganded GR in NSPCs?*

Unliganded GR was recently shown to be necessary for induction of the tumor suppressor gene, BRCA1, in nonmalignant mammary cells (Ritter et al., 2012). GR interacts with the promoter of BRCA1 only in the absence of GCs, while exposure to hormone inhibits this interaction. BRCA1 is expressed in the VZ of the E11.5 mouse telencephalon (including the hippocampal and olfactory bulb primordial) and regulates the proliferation of early progenitors in the E11.5 VZ (Pulvers and Huttner, 2009). Therefore, the unliganded GR may regulate the survival and/or proliferation of VZ progenitors in the dorsal telencephalon through an analogous influence on BRCA1 gene expression or of other transcription factors.

Phosphorylation of GR is a key mechanism for controlling the receptor's activity and can influence the transcriptional targets of the receptor (Galliher-Beckley and Cidlowski, 2009). Interestingly, ligand-independent GR phosphorylation by p38 MAPK occurs *in vitro* on human and rat GR (Galliher-Beckley et al., 2011) and increases the association of GR with its cofactor 14-3-3zeta to change the transcriptional response to hormone. p38 MAPK and other components of the JNK pathway are highly expressed in the intermediate zone (IZ), preplate and cortical plate of the developing mouse dorsal telencephalon, sites where we observed a predominant

nuclear GR localization. Constitutive expression of an upstream activator of the JNK pathway, MUK, from E13, arrests the migration of newborn neurons out of the SVZ/IZ boundary on E16 (Hirai et al., 2002), an effect associated with the microtubule network. Therefore region and neurodevelopmental stage-specific activation of MAPKs that target GR could ultimately influence the subcellular localization of the receptor and its association with unique targets that could differentially impact NSPC function.

2.4.4 *GR and Perinatal GC Use*

While GCs have been used for decades in perinatal medicine, evidence from animal and clinical studies suggest a need to re-evaluate this usage. Early *in vivo* studies with the rhesus monkey from Uno et al., (1990, 1994), demonstrated that prenatal DEX exposure can have deleterious effects on the developing telencephalon architecture, such as reduced cortical size and disrupted lamination of the cortex and hippocampus. Similar results have been obtained in murine models (Fukumoto et al., 2009; Heine and Rowitch, 2009; Khozhai and Otellin, 2008). In humans, antenatal GCs have been linked with reduced head circumference and cortical convolution (Modi et al., 2001; Thorp et al., 2002). In addition, prenatal stress has been linked to attention and learning deficits, anxiety and depression in the offspring (Mesquita et al., 2009). Therefore, when studying the effects of GCs on the developing brain, knowledge of the precise spatial and temporal expression of GR protein is essential to ascertain the molecular basis for potential detrimental effects of antenatal GCs. For example, we observed that GR is highly expressed in the Ctip2-positive subpopulation of layer V neurons of the cortex. These cells are subcerebral projection neurons which extend their axons to the basal ganglia, diencephalon, midbrain, hindbrain, and the spinal cord (McKenna et al., 2011). Hence, they act as efferents for top-down

information transmission, from the cortex to lower order processing centers. Furthermore, we observed that GR expression was high throughout the rostrocaudal extent in layer V and in frontal regions of the cortex, including the prefrontal cortex. Neuronal circuit malfunction in these areas and especially in the prefrontal cortex has been associated with the evolution of neuropsychiatric disorders of which the root is thought to be in the neurodevelopmental period (Tekin and Cummings, 2002). Particularly important in relation to neuropsychiatric disorders is the presence of GR in NSPCs of the GEs. The GEs give birth to interneurons, which populate the telencephalon, hippocampus and olfactory bulb. Interneuronal function and stress i.e., increased *in utero* GC levels have been linked with the risk of developing schizophrenia (Czeh et al., 2005).

In summary, we have shown that GR protein is present in early progenitors of the dorsal and ventral telencephalon at E11.5 and primarily occupied the nucleus of the cells. Moreover, the region-specific expression of GR protein in the developing telencephalon suggests that subcellular localization of the receptor may be subjected to region and neurodevelopmental stage-specific regulation. The results from our study may provide a basis to better understand the effects of GCs on telencephalic development both *in vivo* and *in vitro.* Furthermore, the presence of GR in the nucleus of most NSPCs and its dynamic expression within the neuroepithelium raises a question about the precise role of GR in NSPCs, particularly in the absence of endogenous GC production. A more in depth understanding of GR's expression dynamics in the developing brain may provide insights relevant for the limitation of potential neurologic side effects of GCs, while maintaining and enhancing their beneficial potential during pregnancy.

3.0 SECOND CHAPTER

PREMATURE ACTIVATION OF GR DURING MIDGESTATION ALTERS NEURAL PROGENITOR PROLIFERATION IN A CELL CYCLE DEPENDENT-MANNER AND IMPAIRS CORTICAL DEVELOPMENT

3.1 INTRODUCTION

Synthetic glucocorticoids (sGCs) have been used now more than half a century in preterm medicine mainly in the case of an imminent threat for preterm labor. GCs are naturally organ growth promoting hormones and in this case are used to help the maturation of the fetal lungs and reduce the incidence of respiratory distress syndrome, periventricular leukomalacia and of intraventricular hemorrhage (Braun et al., 2013; Pang et al., 2012; Tegethoff et al., 2009). Nonetheless, approximately 25-30% of pregnancies at risk for preterm labor treated with sGCs reach term (Davis et al., 2009). Dexamethasone (DEX), a potent sGC, is the suggested GC to be used for preterm labor between 24-34 weeks, according to the WHO guidelines. DEX is resistant to 11β-HSD2 placental degradation and therefore accumulates at much higher levels in fetuses. During the critical human gestational ages of 24-34 weeks allowed for DEX treatment, and especially from 30 weeks and onwards, the embryonic brain undergoes a dramatic spur of development, mostly in terms of cortical surface expansion and development of the elaborate gyrification pattern of the cortex (Kapellou et al., 2006). Between weeks 30-32, the fetus starts to produce *de novo* GCs which stimulate lung surfactant production in preparation for labor (Braun et al., 2013). Otherwise, before this period the levels of endogenous GCs (i.e. cortisol in humans) in the fetus are relatively low, due to minimal *de novo* production and minimal contributions from the mother because of efficient cortisol metabolism by placental 11ß-HSD2 (Ishimoto and Jaffe, 2011). Thus during the period that DEX is typically given to pregnant women, the brain is considered a GC-poor environment, suggesting that interaction of DEX with the brain GR will be premature. Accumulating evidence both from animal and clinical studies are revealing that exposure to sGCs during this critical fetal period may result to changes in cortical development which can be permanent and may manifest postnatally as cognitive, behavioral and affective

problems (Huang, 2011; Lupien et al., 2009; McEwen, 2005). Studies in different animal models such as rodents, sheep (in which the time of fetal lung development mimics closely the human) and primate, suggest that sGCs can affect cortical, cerebellar and hippocampal development (Khozhai and Otellin, 2008; Sundberg et al., 2006; Uno et al., 1990; Uno et al., 1994) impair cerebral myelination and change the normal course of development of different neurotransmitter systems such as serotonin and dopamine (Kreider et al., 2006). Moreover, through premature initiation of the GC-GR signaling cascade, the function of the HPA axis is altered, leading to a predisposition for a multitude of medical conditions ranging from metabolic, cardiovascular to affective and even neuropsychiatric problems (Huang, 2011). Accumulating results from children that had been prenatally exposed to sGCs but born at term, show that GC exposure may lead to reduced birth weight and head circumference, and importantly changes in the cortical structure that are associated with the development of affective problems (Braun et al., 2013; Davis et al., 2013). Therefore, these results point to a need for in-depth analysis of the role of the GR in cortical development and call for an understanding of the effects of activating prematurely the GR-GC signaling cascade.

In this study we sought to examine the effects of premature activation of the GR-GC signaling on cortical development by specifically dissecting the cell-specific effects of prenatal DEX exposure on the different neural progenitor populations of the cortex and on the development and cytoarchitectural composition of the developing cortex. To our knowledge this is the first study using this approach, since none of existing studies has focused on this level of detail on the effects of the premature activation of the DEX -GR signaling in the cortical progenitor cell biology and on cortical cytoarchitecture. Specifically, we used a single course of DEX at a clinically relevant dose in E14.5 mice (midgestation) to examine the effects of a

premature GR activation on the proliferation of RGCs and IPCs. My results show that DEX-induced GR activation shifts the balance towards neuronogenesis at the expense of proliferation by primarily affecting RGC proliferation. This effect was S-phase dependent, since in RGCs that were in S-phase, DEX promoted an expansion of the neurogenic pool and in S-phase IPCs, DEX promoted neurogenic divisions. In non-S phase RGCs DEX exposure promoted an IPC fate. Within this context, BrdU birthdating 72 hours following the DEX injection on E14.5 (E17.5), revealed that a higher proportion of BrdU cells was located in the cortical plate and a smaller proportion was found in the progenitor domain of DEX-treated embryos, compared to the controls. Moreover, DEX exposed animals had increased numbers of neurons in all layers of the cortex (LVI-II) but had a smaller cortex in terms of cortical thickness and surface and an impaired brain growth overall as revealed by reduced brain to body weight ratio. These data support the hypothesis that DEX promoted neuronogenesis at the expense of proliferation. Collectively our results indicate that premature DEX-induced GR signaling during mid-neurogenesis, alters NPC proliferation and leads to long-term changes in the cortical cytoarchitecture highlighting a critical role for the unliganded GR in neural development which is disrupted in the presence of DEX.

3.2 EXPERIMENTAL DESIGN

3.2.1 Animals

Timed-pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC) and pregnant dams were housed individually and received chow and tap water ad libitum. The day of vaginal plug was designated as E0.5. Animal protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and adhered to the National Institutes of Health guidelines.

3.2.2 Tissue Preparation and Processing

Embryos were collected via caesarian section and were fixed in 4% paraformaldehyde (PFA; pH 7.4) and processed through increasing sucrose gradients for cryosectioning. Subsequently, brains were embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T.) compound (EMS, Hatfield, PA) and sectioned at 20 μm. Alternatively for the production of paraffin sections, embryos were fixed overnight at 4^{o}C in fresh SEARS solution (30% formalin; 10% glacial acetic acid; 60% pure ethanol) and processed for paraffin embedding and sectioned at 10 μm. For each experimental condition at least 3 animals were used.

3.2.3 Immunohistochemistry

Immunohistochemistry for cell-markers was applied as described previously (Tsiarli et al. 2013). Briefly, cryosections were dried for 30 minutes at 46^{o}C or air-dried at room temperature for approximately an hour and then washed with 0.1% Triton-PBS. Paraffin sections were warmed at 56^{o}C and then de-paraffinized and rehydrated through a series of xylene and decreasing

concentrations of ethanols (100%, 95%, 70%), rinsed with water and washed with 0.1% Triton-PBS. Antigen retrieval was performed with same-day-made 1X sodium citrate buffer (2.194 gr sodium citrate in 1 L PBS, pH 6.0) by microwaving for all staining combinations. After reaching room temperature, the sections were blocked with 10% heat-inactivated normal goat serum (HINGS, Jackson ImmunoResearch, West Grove, PA), for 1 hour at room temperature, followed by three overnight incubations at 4°C with the primary antibody, which was diluted in blocking solution. Sections were subsequently incubated for 1 hour in the secondary antibody at room temperature, rinsed and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Table 3.1 describes the list of primary and secondary antibodies used in this study. Fluorescent images were visualized with an Olympus Fluoview 1000 confocal microscope (single optical sections 2.5 um thickness, 40X oil immersion) or a on a Nikon fluorescent microscope, photographed with a Photometrics CoolSNAP digital camera and visualized with the NIS elements software (Nikon). Images were adjusted in Photoshop CS5.

3.2.4 DEX and BrdU injections

Timed-pregnant mice at E14.5 were intraperitonealy (ip) exposed to a single course of DEX (0.4 mg/kg) or vehicle. Immediately after, animals were given a single ip pulse of BrdU (0.5 mg/kg). Embryos were collected by cesarean section, sacrificed by decapitation and studied after 24 or 72 hours after DEX injection (E15.5 and E17.5 respectively).

3.2.5 Fluorescence-Activated Cell Sorting (FACS) Analysis

Dorsal cortex tissue was dissected in cold PBS following 24 hours of DEX and BrdU exposure. The tissue was dissociated in PBS using a 200 μl pipette and gentle aspiration, filtered through a 40 μm filter and pelleted at 200 rpm for 5 minutes, at 4°C. Following this step, cells were washed with cold PBS and while vortexing 70% Ethanol in PBS was added drop-wise to avoid clumping and cells were left to fix for 30-60 minutes at 4°C. After fixation, samples were pelleted as above and the ethanol was carefully washed by cold PBS. Antigen retrieval was performed by adding distilled water and then dropwise 12N HCL until diluted to 2N and left for 30 minutes in room temperature (RT). After antigen retrieval, cells were pelleted and washed with PBS and then blocked with 10% HINGs for 20 minutes in RT. Then samples were stained with BrdU-rat in 10% HINGS (1:50, Abcam) approximately for 30 minutes in RT, pelleted, rinsed with PBS and incubated with Alexa 647 anti-rat (1:1000, Invitrogen), and after with DAPI to stain DNA. Flow cytometry, was performed at the University of Pittsburgh Immunology Flow Cytometry Core, using an LSRFortessa cytometer. Parameter adjustment and analysis of the raw FACs data was performed by Dr. Albert D. Donnenberg and Mr. Michael Meyer at the Cell Cytometry Facility of the University of Pittsburgh Cancer Institute (UPCI) which is supported in part by award P30CA047904.

3.2.6 Quantification and Statistical Analysis

All statistical data are presented as mean ± SD. Data were tested for statistical significance by the two-tailed Student's t test using the GraphPad Prism software 6 (©2014 GraphPad Software, Inc., CA, USA), at a significance level of 0.05, using an $n \geq 3$ per experimental condition.

Table 3. List of Antibodies and Dilutions

Primary Antibodies	Secondary Antibodies
BrdU, Abcam rat: 1:50 sheep: 1:50	AlexaFluors, Invitrogen All used @1:1000
Cux1, Abcam rabbit: 1:100	A488 anti-rabbit anti-sheep
Tbr1, Abcam rabbit: 1:100	A647 anti-rat anti-mouse
Ctip2, Abcam rat: 1:500	A555 anti-mouse
Tbr2, Abcam rabbit: 1:500	A568 anti-rat
Satb2, Abcam mouse: 1:200	

3.3 RESULTS

3.3.1 A single exposure to DEX during mid-gestation (i.e. E14.5) alters brain development when assessed at E17.5.

Several clinical studies examining the effects of antenatal GCs given for preterm labor, have reported adverse effects on birth weight, head circumference and later neurological development of the offspring. These reports also include term-born children that were exposed prenatally to GCs (Davis et al., 2009; Davis et al., 2013; Khalife et al., 2013) who therefore do not have complications associated with premature birth and neonatal intensive care. These results have been replicated in different animal models such as in the mouse, rat, monkey and sheep (Braun et al., 2013; Tegethoff et al., 2009). Nevertheless, due to the fact that some studies use multiple courses of DEX and at different developmental stages, some results are conflicting. In order to identify the biological consequences of a single course of DEX in a clinically relevant dose on brain development, we exposed midgestation (E14.5) embryos to 0.4 mg/kg DEX and collected the embryos 72 hours after the injection (E17.5). Our experimental setting is unique due to two reasons, firstly because we are using a single exposure to DEX which allows us to examine the "baseline" effects of DEX on cortical development, without having the ambiguity of multiple injections. Secondly and most importantly, these settings mimic the human fetal period during which DEX is administered, where endogenous levels of GCs are low. Similarly, E14.5 mouse embryos have minimal levels of endogenous GCs. Thus this allows us to dissect the effects of a premature DEX-induced activation of GR signaling on neurogenesis and cortical development.

For all the injections in these study, the timed-pregnant dams were injected between 10:00-11:00 am and the embryos were collected after the selected time window again between

10-11:00 am. Initial experiments used E18.5 embryos that have a more mature cortical plate, but since this is close to parturition in the mouse, a number of dams were giving birth prior to our planned collection of embryos. Therefore we examined DEX effects at E17.5 and collected controls and treated embryos by caesarean section. To evaluate brain growth following 72 hours after DEX exposure, we analyzed the brain to body weight ratio (BBWR), which provides an indirect index of brain growth. The total weight of the embryos was first recorded, followed by the weight of the whole brain. In agreement to findings of other studies and to our hypothesis that DEX would alter brain growth, in DEX treated animals the BBWR was reduced compared to the controls (Fig. 19A). The reduction was evident even after controlling for litter size (Litter A: $n_{control}$ = 13, BBWR=; n_{DEX} = 6; Litter B: $n_{control}$ = 11; n_{DEX} = 12). Therefore, an average reduction of BBWR was observed for both DEX litters at 15% over the controls (pooled results from two litters). Interestingly, in both experiments, the body weight was slightly but significantly increased (Appendix B, Figure 37; mean (g) ± SD; Litter B: Control 1.2 ± 0.075; DEX 1.4 ± 0.086; $p < 0.0001$), suggesting that DEX differentially affected the body growth versus the brain. Despite this fact, normalized BBWR was reduced, indicating that the brain does not grow proportionally to the body when it is exposed to DEX. This result suggests that a single course of DEX during E14.5 leads to an impairment in body and brain growth.

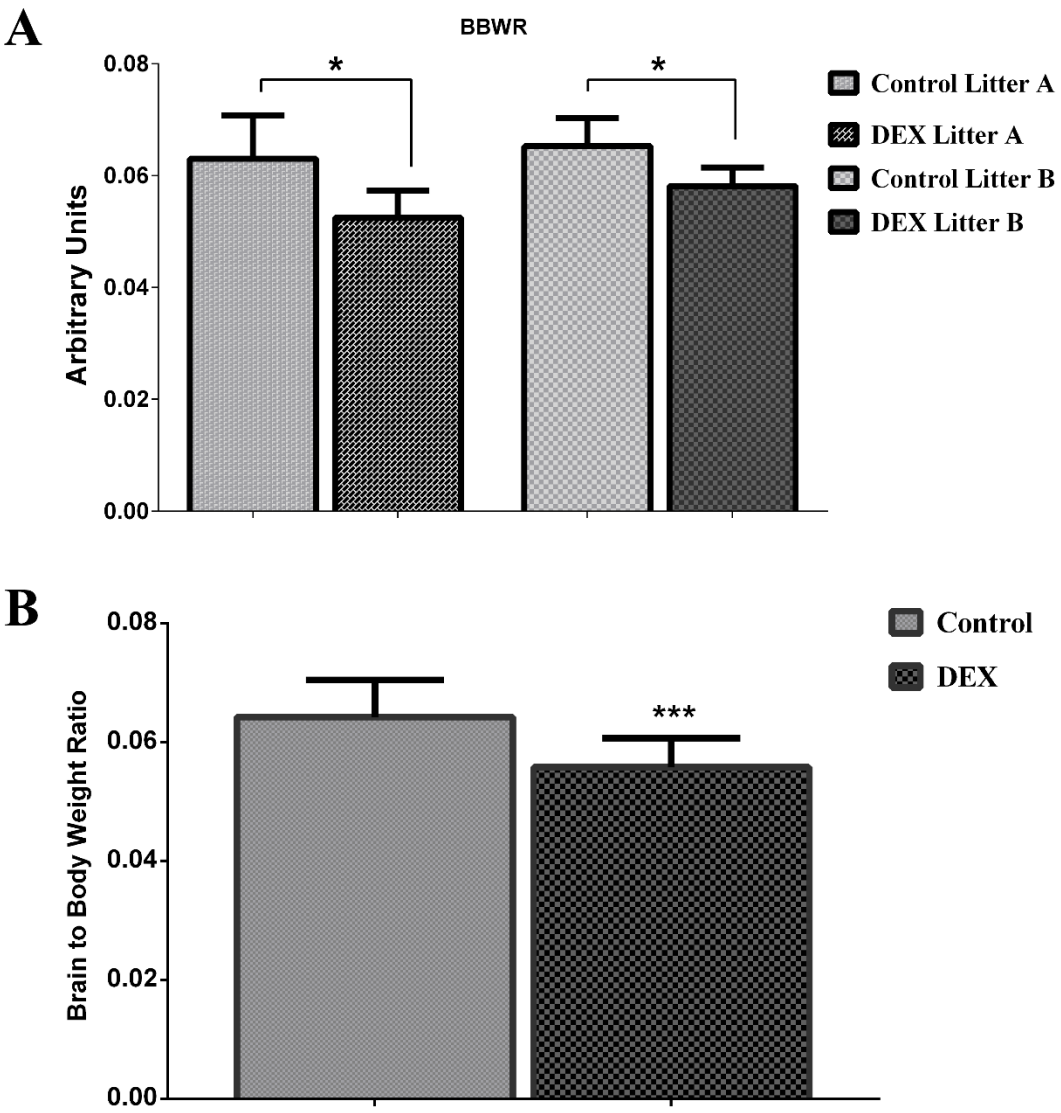


Figure 19. A single DEX course on E14.5 impairs brain growth by E17.5 as evaluated by the Brain to Body Weight Ratio (BBWR). ***A***. BBWR for two separate groups of litters, of different litter sizes, revealed that DEX effects are independent of litter size. BBWR mean ± SD; Student's t-test, Littter A: $n_{control}$ = 13, n_{DEX} = 6, Control_a BBWR= 0.063± 0.0078 (analyzed 6 animals), DEX_a BBWR= 0.053 ± 0.0048; $p< 0.05$; Litter B: $n_{control}$ = 11; n_{DEX} = 12 (analyzed 7 per group); Control_b BBWR 0.065± 0.0050, DEX_b 0.058± 0.0033, $p< 0.05$. ***B.*** Pooled data from both litters, Control BBWR 0.064± 0.0063, DEX BBWR 0.056 ± 0.0049, $p <0.001$.

3.3.2 Activation of GR-GC signaling by a single course of DEX on E14.5 causes a reduction, both in the radial and lateral expansion of the cortex after 72 hours.

Cortical development proceeds in the radial dimension resulting in a progressive increase in cortical thickness (radial expansion), and on the lateral dimension (lateral expansion) resulting in an expansion of the cortical surface. During the 24-34 week time-window when DEX is administered to pregnant women, the brain undergoes dramatic development both in terms of cortical surface increase and in terms of increasing complexity in the cortical folding pattern (Dubois et al., 2008; Kapellou et al., 2006). Not surprisingly, antenatal GCs have been reported to be associated with a reduction in cortical surface and cortical convolution complexity (Modi et al., 2001), and also with reduction in cortical thickness, even in term born children (Davis et al., 2013). Therefore it seemed plausible that the impairment of brain growth we observed in DEX-treated embryos may be the result of an underlying impairment in cortical development. Following the method described by Pulvers and Huttner (2009), we analyzed both the cortical surface and cortical thickness in Nissl stained E17.5 coronal sections of E14.5 DEX-treated and control embryos (Figure 20). Indeed, in DEX-treated animals both the cortical surface was reduced and cortical thickness were significantly reduced by 15% and 10% respectively (Figure 20).

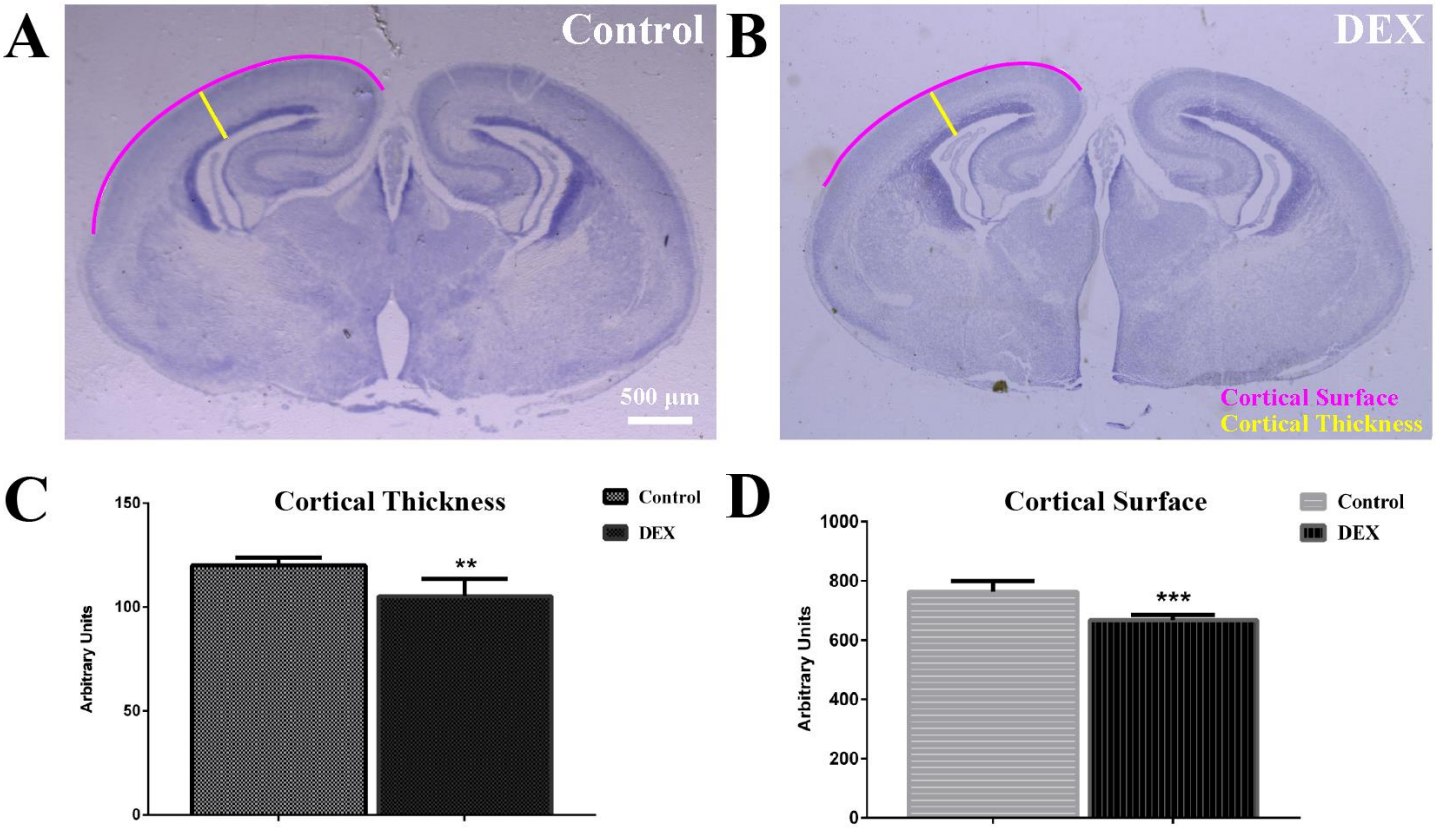


Figure 20. A single exposure to DEX on E14.5 impairs cortical development as seen after 72 hours. ***A, C.*** DEX exposure on E14.5 induces an impairment in the lateral expansion of the cortex i.e., cortical surface, by E17.5 (pink line). Mean ± SD, $n_{control}$ = 5; $n_{DEX\ l}$= 6; Control 763 ± 36, DEX Control 667 ± 18, $p < 0.001$. ***B, D.*** Also the cortical thickness (yellow line) in DEX-treated embryos is reduced Control 120 ± 3.8, DEX Control 105 ± 8.3, and $p < 0.01$.

3.3.3 DEX-induced GR signaling during mid-gestation promotes supernumerary neuron production in deep- and upper-cortical layers.

Since both cortical surface and cortical thickness are a reflection of cortical cytoarchitecture integrity (Fischl et al., 2004), we wondered if the impaired cortical growth of the DEX-treated embryos could be the result of altered cortical lamination. To evaluate this hypothesis we used specific molecular markers to individually label layers VI-II and the subplate (SP). Initially we evaluated deep layers VI and V, since at E14.5, when the embryos are exposed to DEX, layer V production is at its maximal rate, whereas, layer VI production is being completed (Takahashi et al., 1999). Upon initial observations the spatial ordering of the deep cortical layers VI (Tbr1+) and V (Ctip2+) appeared to be preserved (Figure 21). Therefore DEX exposure did not disturb the process by which these layers are specified and established. We observed a similar situation in the case of upper layers IV (Satb2+) and II/III (Cux1+), whereby the cells that already migrated in the cortical plate were at their expected positions whereas, many were still migrating though the intermediate zone and the deep layers (Figures 21C-D'). Therefore, DEX treatment did not affect the spatial positioning of the cortical layers.

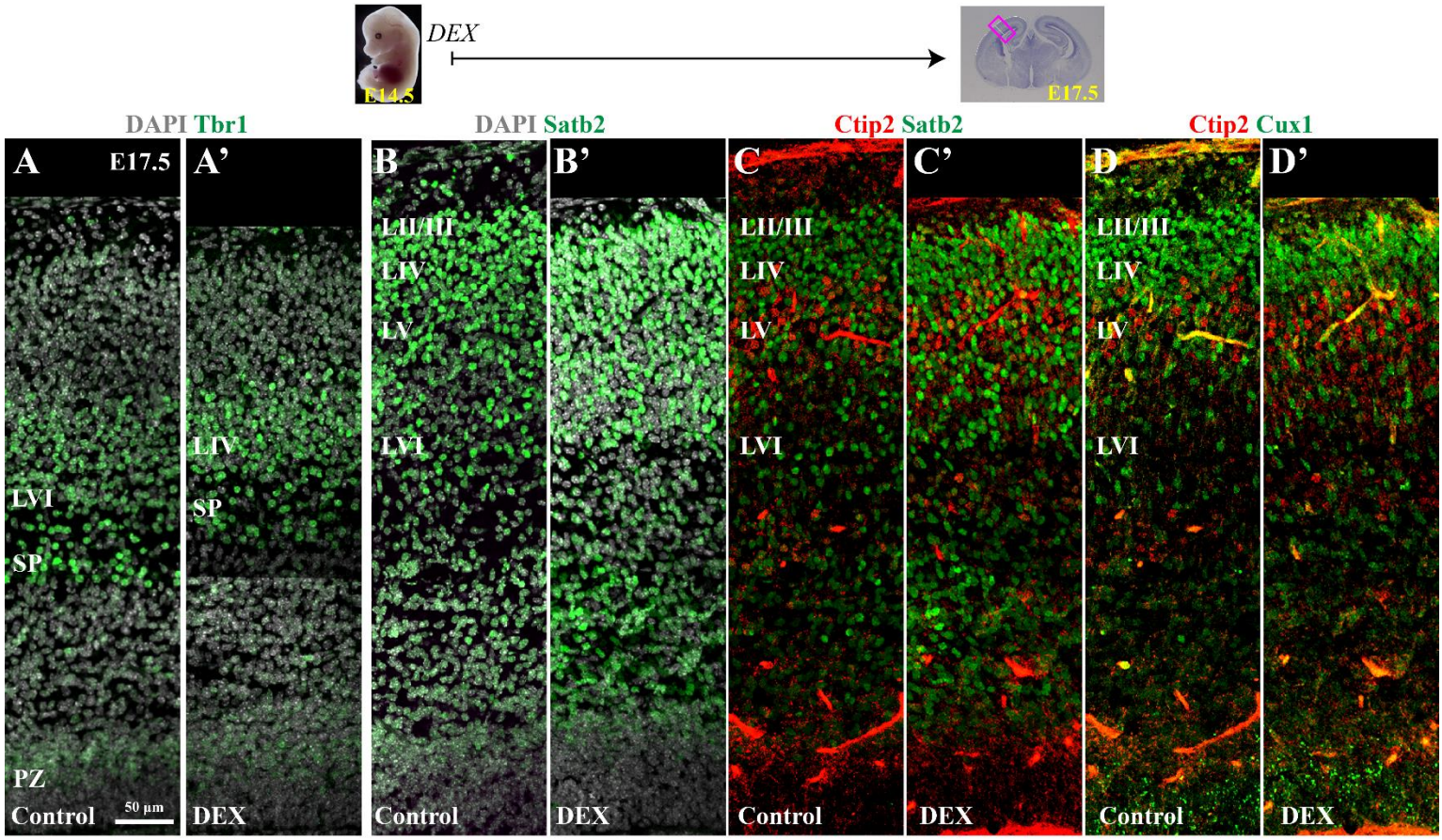


Figure 21. DEX exposure on E14.5 does not affect cortical layer positioning of postmitotic neurons in the E17.5 cortical plate. A-D. The positioning of the layers is relatively similar in the control embryos versus **A'-D',** the DEX-treated embryos. Nevertheless, close inspection of the staining patterns of each marker in the cortical plate especially indicates DEX may have promoted differences in the abundance for each marker. Tbr1 deep layer VI (LVI) and Subplate (SP) marker; Ctip2: LV marker; Satb2: LIII-IV marker; Cux1: LII/III marker. PZ, progenitor zone, Scale bar 50 µm. Embryo image source Zhang Lab, Harvard.

Nevertheless, visual inspection of control and DEX-treated cortical sections co-stained with DAPI and laminar markers such as Satb2 (Figures 21B -B') and/or with laminar markers such as Satb2/Ctip2/Cux1 (Figures 21C-D'), suggested that the number of neurons may differ between the two groups. Therefore, as a first approach to evaluate this observation, we counted the number of DAPI stained cells within the cortical plate (including the subplate). Interestingly, despite having a thinner cortex, DEX-treated embryos also had an increased number of DAPI cells in their cortical plate (Figure 22). This result prompted the question of whether this increase was due to a contribution from a specific layer population. On the day of DEX exposure, i.e., E14.5, the majority of LVI cells is already born and LV is reaching the climax of its birth rate. Also, the generation of upper layers II/IV is steadily increasing from E14.5 onwards (Takahashi et al., 1999). Therefore the most likely candidate to have contributed the increased number of DAPI cells in the cortical plate was layer V, then followed by upper layers II/IV. Hence, with the help of the laminar markers, we quantified the number of neurons that correspond in each layer within the cortical plate, including deep layer VI and the SP populations (Tbr1+) in the analysis. As expected, DEX-treated cortices had a large increase in the number of LV neurons, which amounted to a 64% compared to the control values (number of Ctip2+ cells), and an increase in Satb2+ (32% compared to controls), as well as Cux1+ neurons (43% compared to controls, Figure 23B). On E17.5, many of the neurons of upper layers LIV-II are still migrating as seen in Figures 23A and C, therefore the above percentages are more likely underestimations. Therefore, DEX-induced GR signaling initiated on E14.5, resulted in a global effect on the generation of neurons, without having a subtype-specific effect, since neurons in all cortical layers, including deep layer VI were increased.

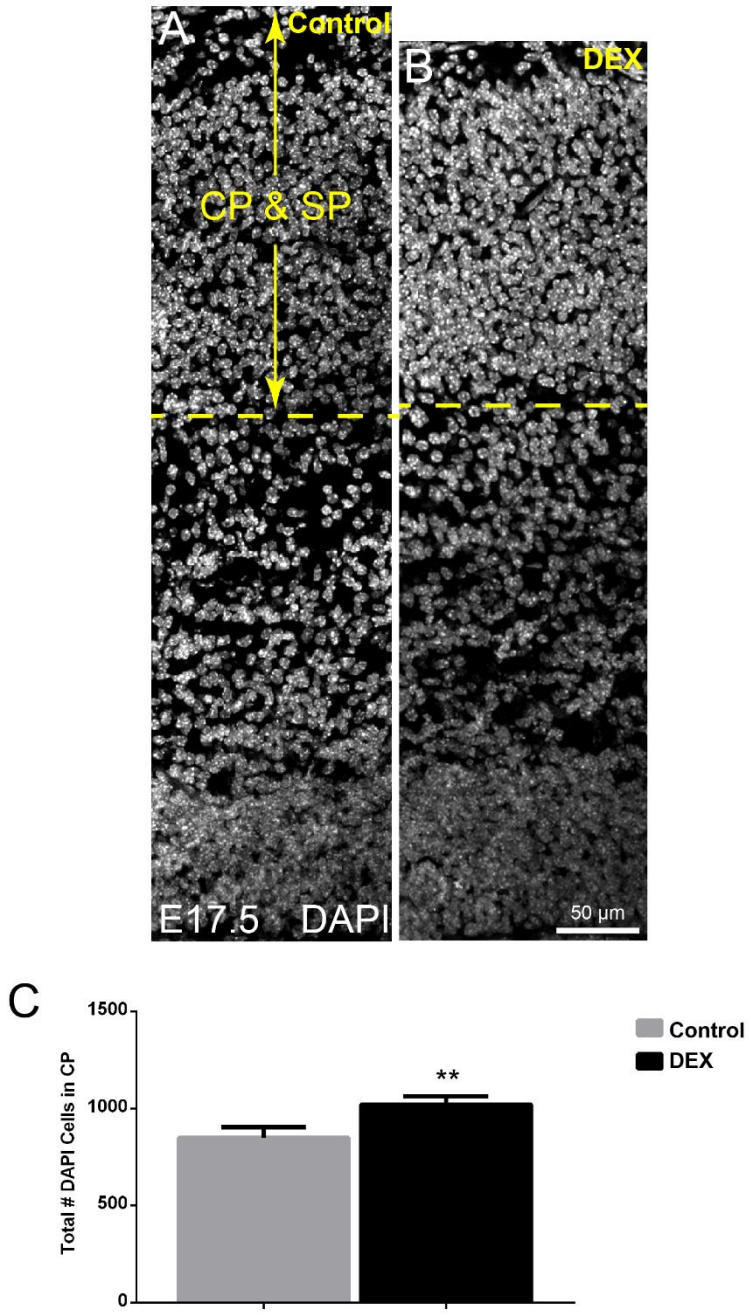


Figure 22. DEX exposure on E14.5 results in higher number of cells within the cortical plate. *A, B.* In the DEX-treated cortices (B) the number of neurons increases by 24% compared to the controls (A). ***C.*** Number of DAPI cells in the cortical plate ± SD, Student's t-test, n= 4; Control 826 ± 29, DEX 1021 ± 44, $p < 0.01$.

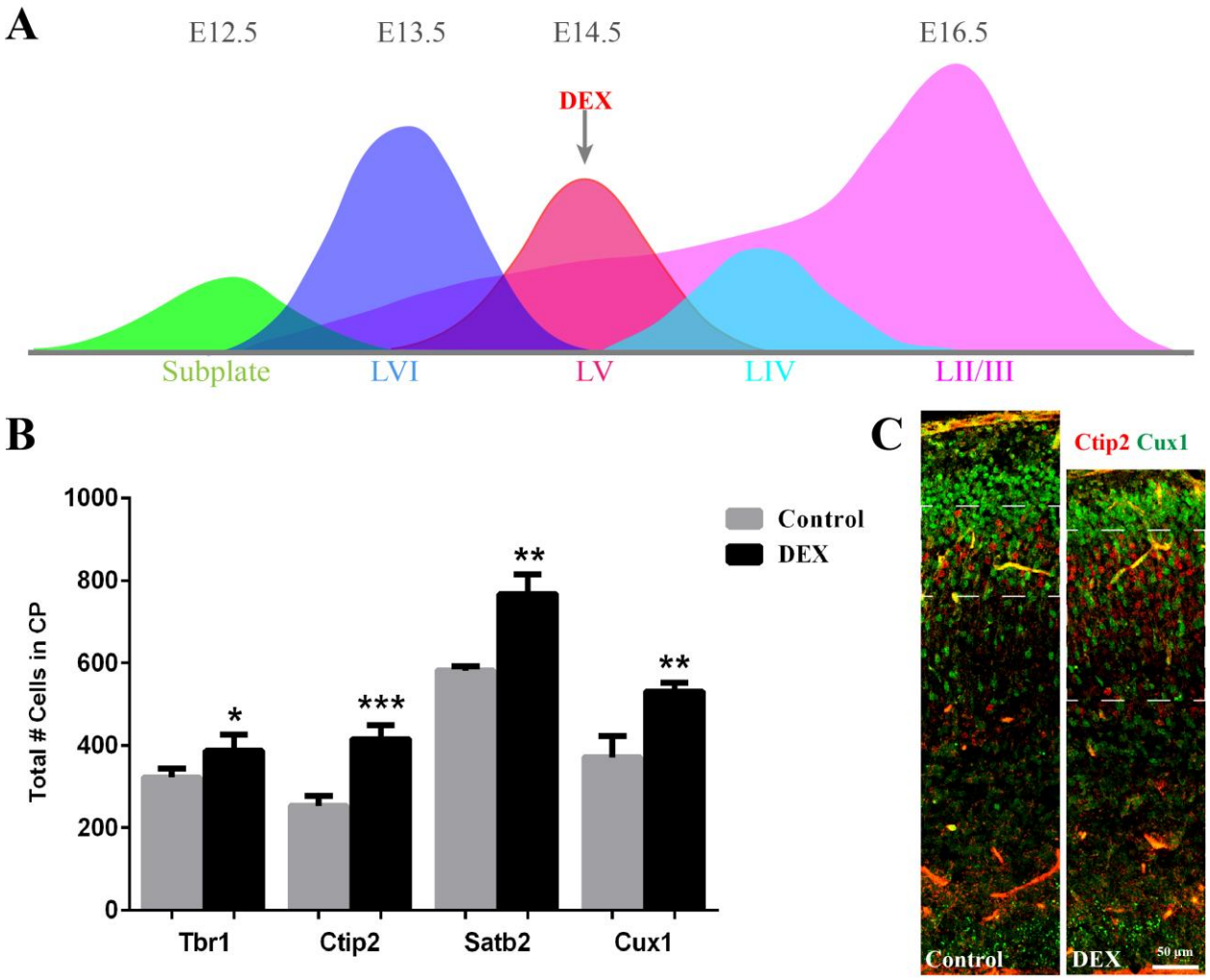


Figure 23. A single exposure to DEX on E14.5 induces a supernumerary production of neurons in all layers including deep layer VI on E17.5. ***A.*** Neuronal production of Layer V (LV) reaches its maximum on E14.5 when DEX is given, while the production of upper layers LII-IV is steadily increasing. ***B.*** DEX exposure causes an increase in the production of neurons in all layers with maximum effect on LV, which increases by 64% compared to the control. Number of Layer Marker-positive cells ± SD, Student's t-test, $n_{Control}$=3, n_{DEX} = 4; Tbr1: Control 322 ±21; DEX 387±39, $p < 0.05$; Ctip2: Control 253 ± 23, DEX 415± 33, $p < 0.001$; Satb2: Control 581 ± 10, DEX 767 ± 47, $p < 0.01$; Cux1: Control 371 ± 52, DEX 530 ± 21, $p < 0.01$). **C.** Ctip2+ LV neurons in the DEX –treated cortices are more and occupy a greater area (outlined region). Scale bar 50 µm.

3.3.4 BrdU birthdating on E14.5, reveals a higher proportion of cells that have migrated in the cortical plate at the expense of progenitor-self renewal in DEX-treated embryos.

The fact that following 72 hours of a single DEX exposure on E14.5, the cortex contains more neurons albeit is reduced in thickness prompted the question of whether this was the result of altered proliferation of the progenitors that generate these neurons. To address this possibility we injected E14.5 mice with DEX and also gave them a pulse of BrdU to label the proliferating population going through S-phase. Then we analyzed the number of BrdU+ cells in the cortical wall after 72 hours. This strategy allowed us to trace the number and position of the progeny of NSPCs that were in S-phase during the time that DEX induced GR signaling. Interestingly, DEX treatment at E14.5 caused a 39% increase in the number of BrdU labelled cells occupying the cortical wall by E17.5 (Figure 24). Visual inspection of the BrdU pattern throughout the cortical wall suggested that the distribution of the BrdU+ cells differed between controls and DEX-treated embryos (Figure 24). Thus, to determine whether this pattern reflected a change in the post-mitotic output of the NSPCs following DEX exposure, we evaluated the proportional distribution of the BrdU+ cells throughout the functional zones of the cortex i.e., the cortical plate (CP), subplate (SP), intermediate zone (IZ) and the progenitor zone (PZ). In agreement to the increase in neurons of all layers that we had observed previously, DEX-treated cortices contained a greater proportion of BrdU+ cells in the CP (+19%) (Figure 25B) specifically in LV-II (Figure 25C), but had a smaller proportion of BrdU+ cells within the PZ (-15%) and the IZ (-16%) (Figures 25B and C).

Collectively, these results suggest that DEX-induced GR signaling on E14.5, promoted progression of NSPCs through the S-phase at E14.5 which was reflected in an increased number of BrdU+ cells. This effect is translated as increased neurogenesis that occurs at the expense of self-renewal of progenitors by E17.5, since a significantly higher proportion of BrdU+ cells occupied the CP and a lower proportion resided in the PZ of the DEX-treated embryos.

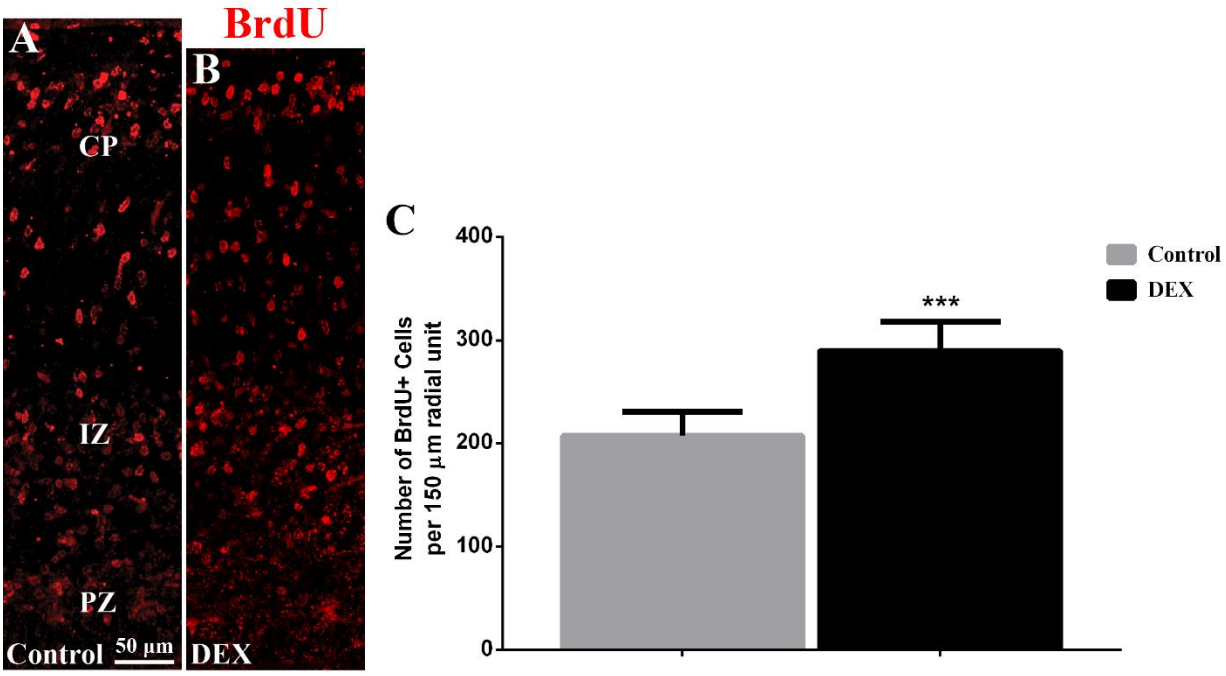


Figure 24. NSPCs exposed to a single course of DEX on E14.5, generate higher numbers of progeny as seen on E17.5. ***A-C.*** S-phase NSPCs that were exposed to DEX on E14.5, generated 39% more progeny compared to controls as seen by the number of BrdU+ cells in the cortical wall on E17.5. Number of BrdU-positive cells ± SD, Student's t-test, n = 6; Control 208 ± 23, DEX 290 ± 28, $p < 0.001$.

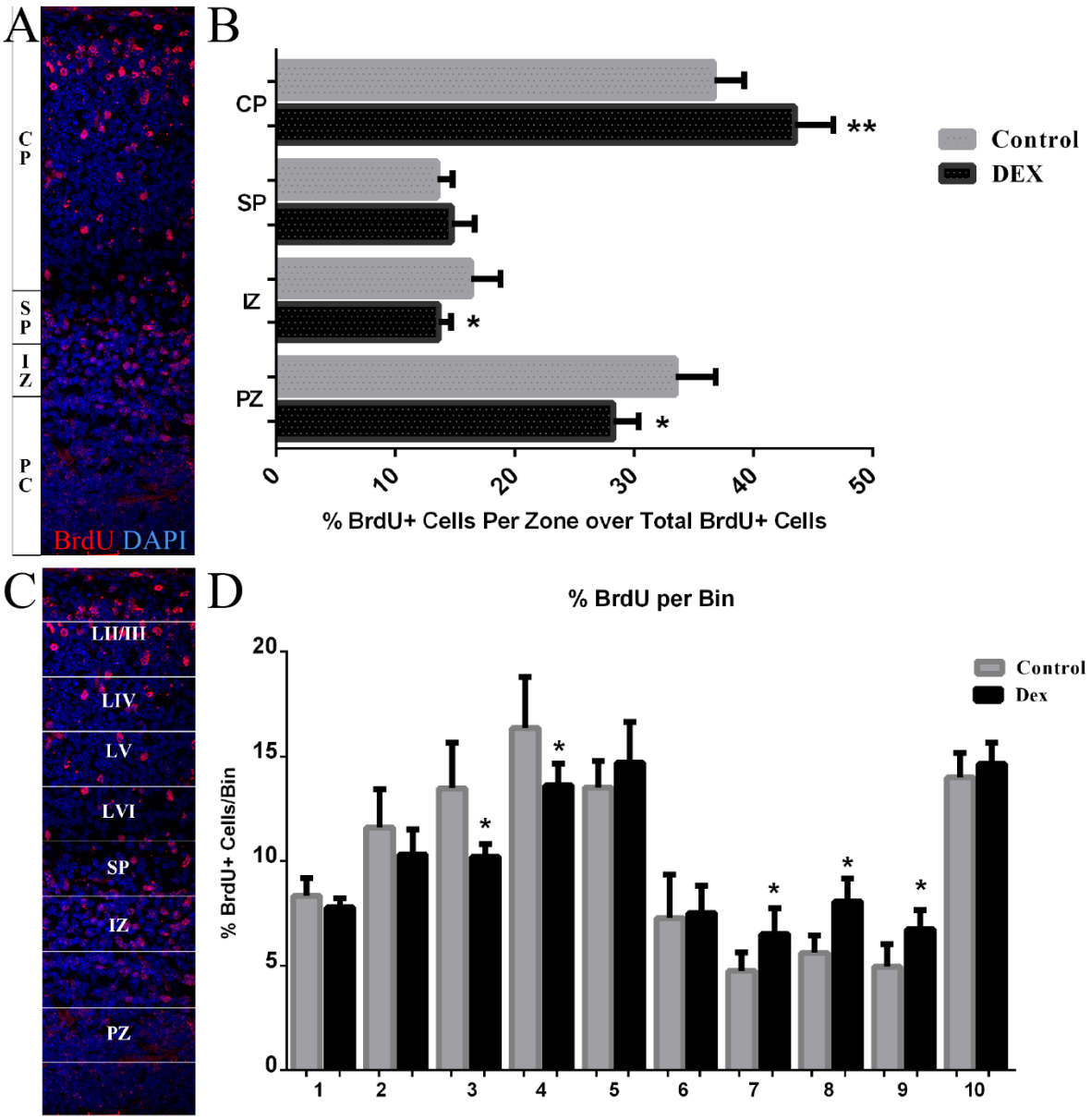


Figure 25. S-phase NSPCs exposed to DEX on E14.5 undergo precocious neurogenesis and generate a higher proportion of neuronal progeny that populate the plate on E17.5. ***A, B.*** A higher proportion of BrdU-labelled progeny of E14.5 NSPCs populate the cortical plate on E17.5. Contrary, a smaller proportion of BrdU+ cells are found in the progenitor zone (PZ) of the DEX–treated embryos. % BrdU-positive cells per zone over total BrdU ± SD, Student's t-test, n = 6; CP: Control 208 ± 23, DEX 290 ± 28, $p < 0.001$.

3.3.5 A single DEX exposure on E14.5 increases the number of NSPCs progressing through S-phase.

Midgestation is a time of critical changes in the neurogenetic process, whereby the cortical plate increases in size as a result of the neurons generated from RGCs and IPCs. At this age, neurogenesis reaches its maximal (Sun and Hevner, 2014). Therefore, we expected that DEX-induced GR signaling on E14.5 would affect neurogenesis. Since we already observed an increase in the NSPCs that had gone through the S-phase (BrdU+) 72 hours after DEX exposure, we examined whether this change would be evident after 24 hours, which is a few hours longer than the average duration of the cell cycle on E14.5 (Takahashi et al., 1994; Takahashi et al., 1995; Takahashi et al., 1997; Takahashi et al., 1999). Thus, E14.5 DEX-treated embryos were given a pulse of BrdU and collected after 24 hours, for cell cycle analysis. In DEX-treated cortices, BrdU+ cells formed a thicker zone within the VZ compared to the controls (Figure 26). In addition, the position of the BrdU+ zone in the basal VZ and SVZ was thicker and approximately 1 cell diameter above the corresponding BrdU+ zone in the controls (Figure 26). Consistent with our findings at E17.5, we observed a 24% increase in the number of BrdU positive cells in DEX-treated embryos compared to the controls after 24 hours (Figure 26). This result was also verified by FACs analysis of dorsal cortices from E14.5 embryos exposed to DEX and BrdU for 24 hours (Figure 26). By using the FACs method the difference in the proportion of BrdU+ cells, rose to 56% (Figure 26). Therefore, these results suggest that DEX-induced GR signaling on E14.5, causes an increase in the proportion of NPSCs going through the S-phase which results in an increased production of neurons.

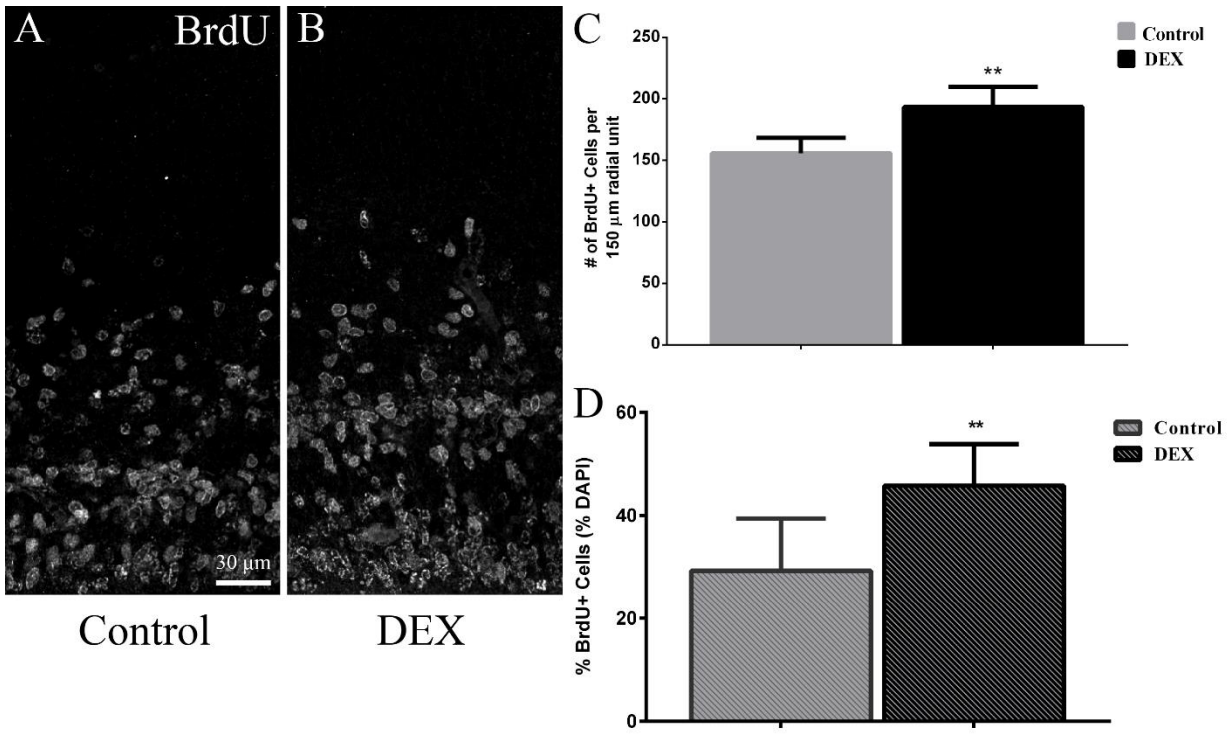


Figure 26. A single DEX exposure on E14.5 increases the number of NSPCs progressing through S-phase after 24 hours. ***A-C.*** The number of BrdU+ cells that progressed through the S-phase, is increased by 24% in DEX-treated cortices. Number of BrdU+ cells ± SD, Student's t-test, $n_{control}$ = 4, n_{DEX} = 6; Control: 156 ± 12, DEX 194 ± 16, p <0.01. ***D.*** FACs analysis of dorsal cortex samples exposed to DEX for 24 hours verified the increase in BrdU: Proportion of BrdU+ cells, Control n=6, 29 ± 10, DEX n=7 49 ± 6, p < 0.01.

3.3.6 DEX induces an increase in the number of IPCS

At mid-gestation the IPC population increases in size (Attardo et al., 2008). Due to the critical contribution of IPCs at this stage and onwards to neuronal production we examined whether this progenitor population was affected by DEX by performing immunofluorescence staining for Tbr2, a specific marker for IPCs. Interestingly, an increase in IPCs was evident in the DEX-treated cortices. Moreover the Tbr2+ cells occupied a wider area due to a wider Tbr2+ VZ zone and a wider bVZ/SVZ zone, which was approximately 1 cell diameter higher and wider compared to the controls (Figure 27). Analysis of the IPC numbers revealed that DEX had promoted a 28% increase in these embryos compared to the controls (Figure 27).

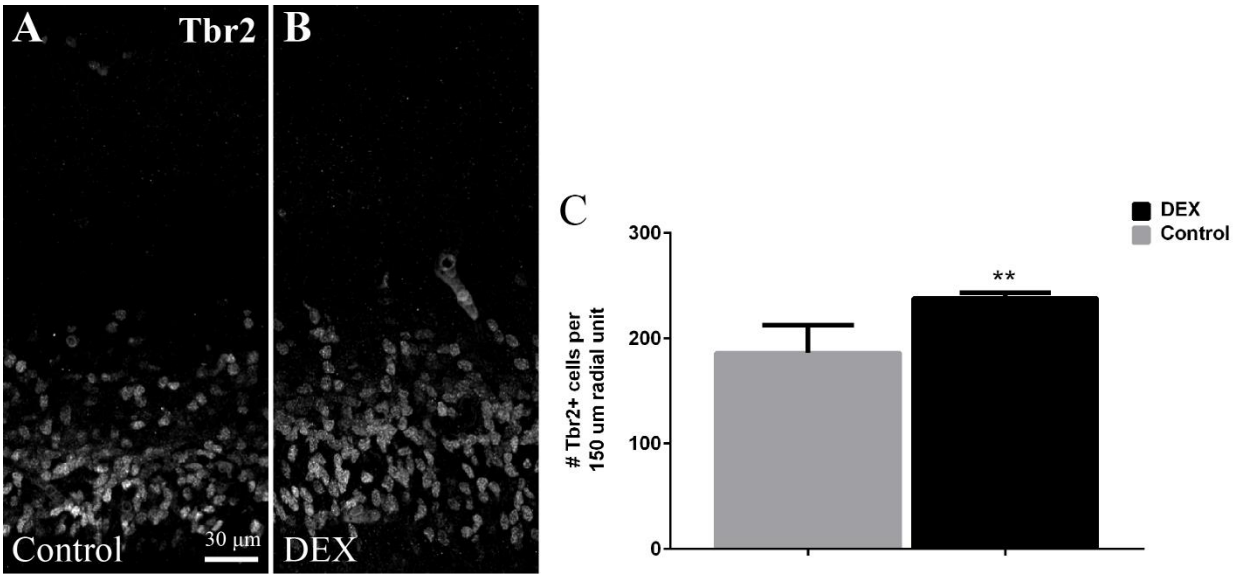


Figure 27. Exposure of E14.5 NSPCs to DEX results in an increase in the number of IPCs after 24 hours. A, B. The number of Tbr2+ cells is increased by 28% in DEX-treated cortices. **C.** Number of Tbr2+ cells ± SD, Student's t-test, $n_{control}$ = 4, n_{DEX}= 6; Control: 186 ± 27, DEX 238 ± 5.5, p <0.01.

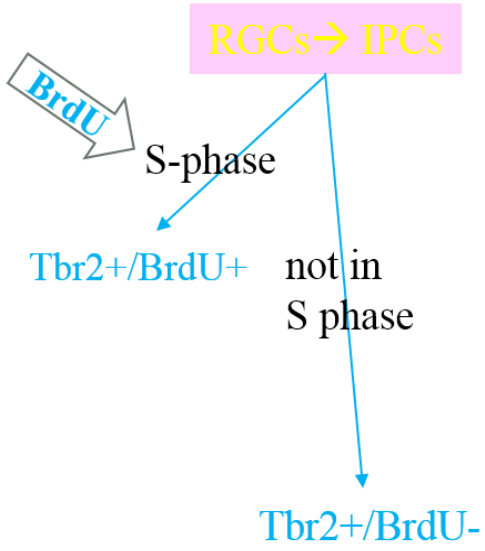


Figure 28. Indirect RGC neurogenic division to IPCs. RGCs can give rise to IPCs by indirect neurogenic divisions by dividing to one RGC and one IPC. If the IPC committed-RGC was passing through S-phase then it would accumulate BrdU+ and give rise to Tbr2+/BrdU+ cells.

3.3.7 Cell cycle phase-dependent effect of DEX on RGC fate.

RGCs divide symmetrically to give rise to two RGCs (self-renewing divisions) or asymmetrically to generate one IPC and one RGC or a neuron and one RGC (Pontious et al., 2008) and Figure 28. Therefore, we wondered whether the increase in BrdU+ cells and Tbr2+ cells following 24 hours of DEX exposure, was a result IPCs generation from NSPCs that went through the S-phase (Englund et al., 2005). Quantification of the number of Tbr2+/BrdU+ cells versus Tb2+/BrdU-, revealed that the increase in the IPC population in the presence of DEX, was actually due to an increase in Tbr2+/BrdU- cells (Figure 29B). Thus, this result suggested that the increased number of IPCs in the DEX-treated cortices were derived from RGCs that had not passed through the S-phase while DEX was acting.

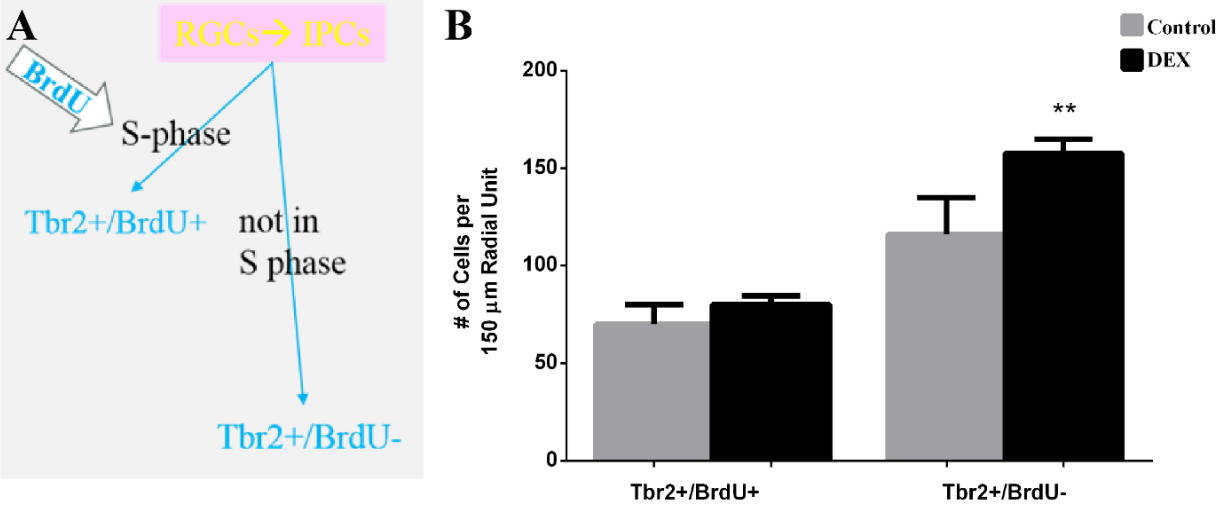


Figure 29. DEX induces IPC fate in RGCs that were not in the S-phase. The number of Tbr2+/BrdU- cells was increased by 32% in the cortices of E14.5 embryos that were exposed to DEX for 24 hours**.** Number of Tbr2+/BrdU- cells ± SD, Student's t-test, $n_{control}$ = 4, n_{DEX}= 6; Control: 116 ± 19, DEX 158 ± 7.3, $p < 0.01$.

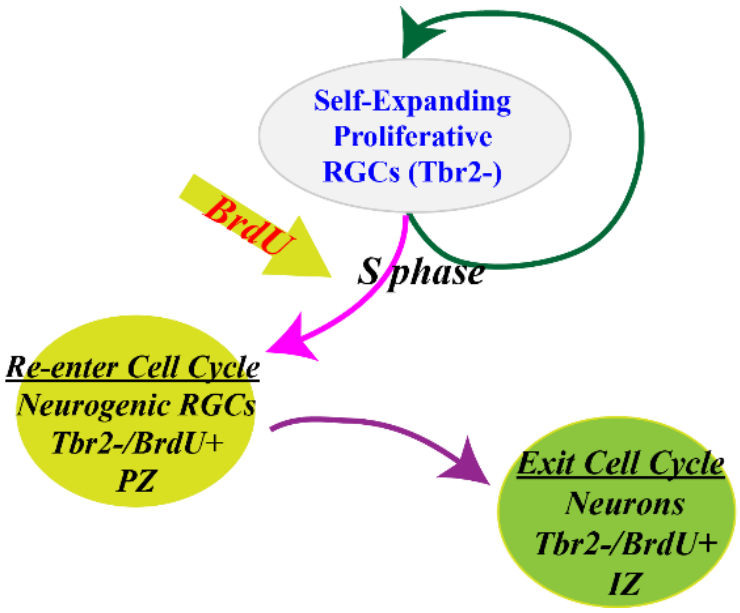


Figure 30. Direct RGC neurogenic divisions. Proliferative RGCs can switch to a neurogenic mode, and re-enter the cell cycle in the progenitor zone (PZ) as neurogenic RGCs which then can divide and give rise to neurons that migrate to the intermediate zone (IZ). If the proliferative RGCs were in S-phase during this transition and exposed to BrdU, then progeny will also contain the BrdU label.

Furthermore, the above result raised the question of what was the fate of NSPCs that went through the S-phase during DEX exposure. Since the majority of progenitor cells in the VZ on E14.5 are RGCs (Arai et al., 2011), counting the number of Tbr2-/BrdU+ cells in the VZ is an indirect way to evaluate S-phase RGCs that have re-entered the cell cycle and remained in the VZ (Re-S-RGCs) (Figure 30). Following this approach we observed a 32% increase in the number of re-entering S-RGCs (Figure 31). Furthermore, we observed a 31% increase in the number of Tbr2-/BrdU+ cells outside of the PZ, which reflect the neurons directly produced by NSPCs in S-phase (Figure 31). Specifically a proportion of these neurons may reflect a contribution from the S-phase RGCs that re-entered the cell-cycle after DEX exposure, and divided giving rise to one neuron and one RGC, since the Tbr2-/BrdU+ cells in the VZ reflect a

mixed population of cycling neurogenic RGCs and their neuronal progeny. In neurogenic IPCs the expression of neuronal markers is present by M-phase, and Tbr2 expression persists at the initial stages of the postmitotic neuron (Englund et al., 2005). Therefore DEX may have also promoted the terminal divisions of neurogenic S-phase IPCs and lead to downregulation of the Tbr2 expression in the postmitotic neurons, which would also add to the increased number of Tbr2-/BrdU+ cells we observed in the IZ.

Overall these results, suggest that DEX promotes neurogenesis by differentially affecting progenitors depending on the phase of the cell cycle. Specifically, in non-S-phase RGCs, DEX promoted the IPC fate (indirect neurogenic effect). In S-phase progenitors, it promoted neurogenesis and specifically by stimulating cell cycle re-entry of S-phase RGCs and promoting acquisition of neurogenic fate (direct neurogenesis). Also, it is likely that in S-phase IPCs DEX promoted their neurogenic divisions and accelerated downregulation of Tbr2 in the postmitotic neurons. Overall, these changes were reflected as an increase in the neurons in the IZ (Tbr2-/BrdU+).

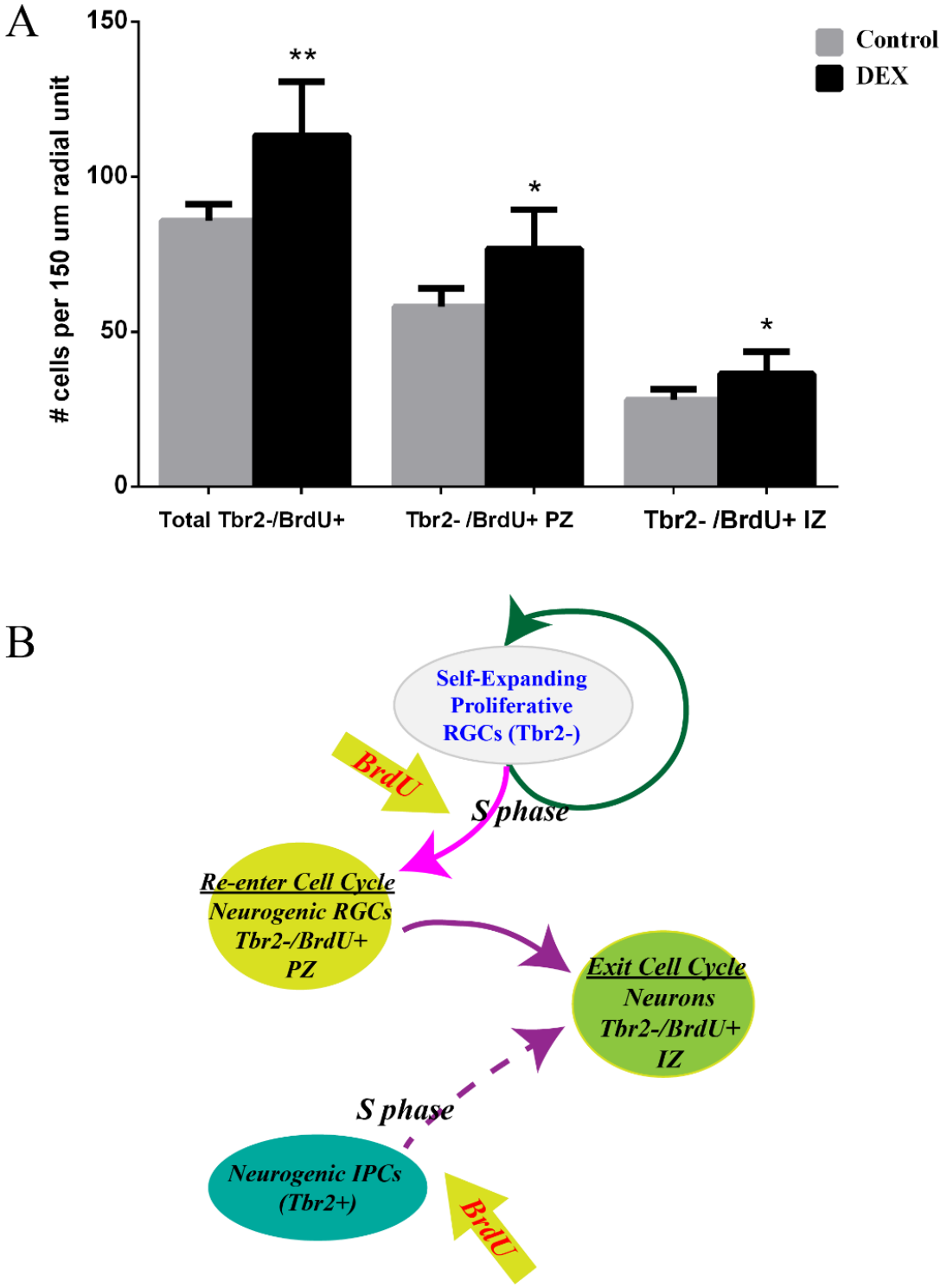


Figure 31. DEX exposure on E14.5 induces precocious neurogenesis in S-phase NSPCs. A. In DEX-treated embryos the number of neurons (Tbr2-/BrdU+) in the IZ is increased by 31%, while the number of S-

phase RGCs that re-entered the cell cycle and are in the progenitor zone (PZ), is increased by 32%, **B,** suggesting that S-phase RGCs (Tbr2-/BrdU+) re-entered the cell cycle as neurogenic progenitors which gave rise to neurons in the 24 hour window. Also, DEX may have promoted and accelerated (dashed arrow) neurogenesis by S-phase IPCs which would also contribute to the increased number of Tbr2-/BrdU+ neurons in the IZ. Number of Tbr2+/BrdU- cells in each zone ± SD, Student's t-test, $n_{control} = 4$, $n_{DEX} = 6$; Tbr2-/BrdU+ Total: Control 86 ± 5.3, DEX 113 ± 17, $p < 0.01$; Tbr2-/BrdU+ PZ: Control 58 ± 5.8, DEX 77 ± 13, $p < 0.05$; Tbr2-/BrdU+ IZ: Control 28 ± 3.5, DEX 36 ± 7.2, $p < 0.05$.

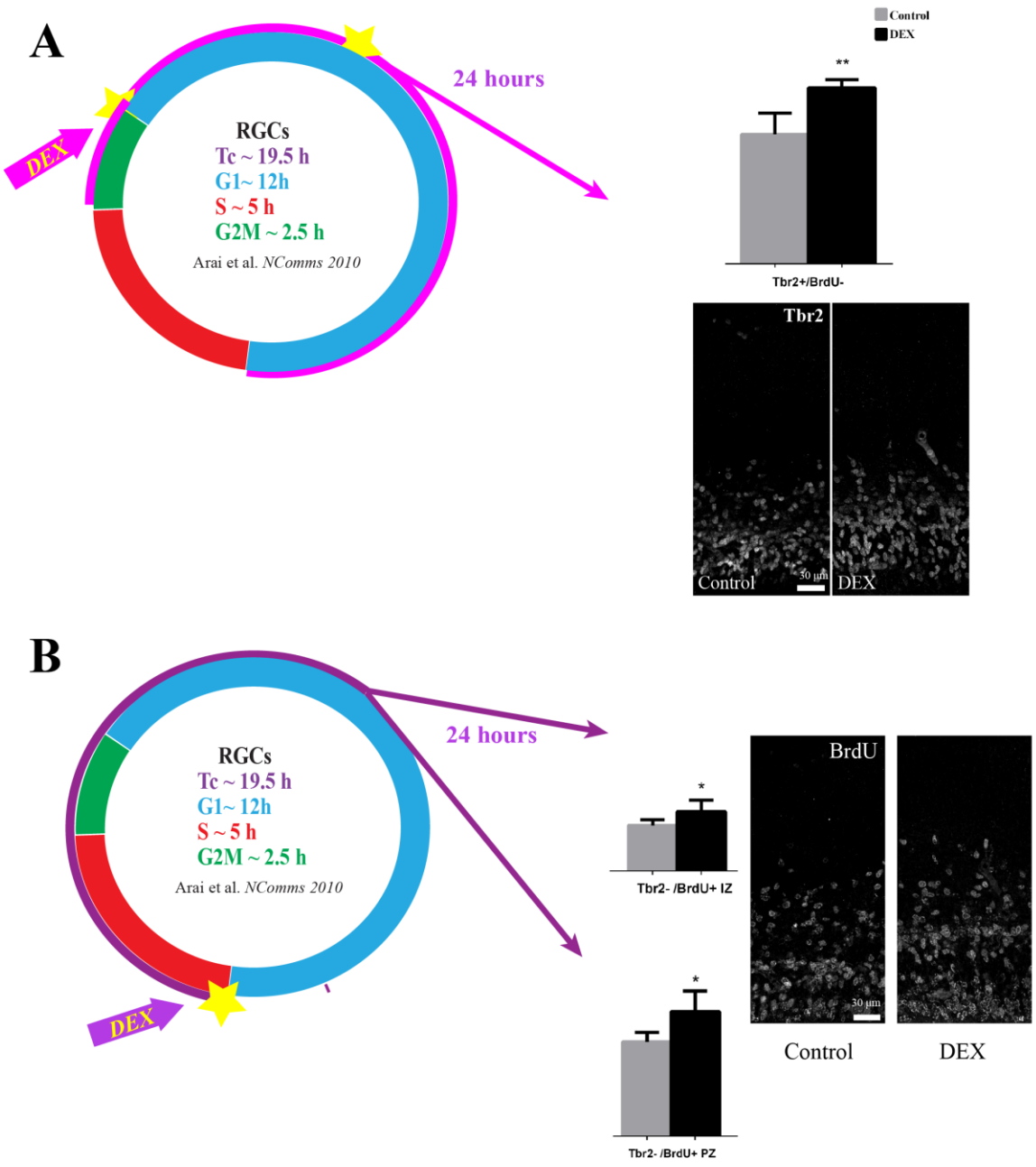


Figure 32. Model for differential effects of DEX on RGC fate depending on the cell cycle fate. **A.** In E14.5 non-S phase RGCs, a 24 hour DEX exposure promotes the IPC fate, reflected as an increase in the number of Tbr2+ cells. **B** In S-phase RGCs DEX promote cell cycle re-entry and direct neurogenesis, reflected in increased number of Tbr2-/BrdU+ cells in the progenitor zone and intermediate zone respectively.

3.3.8 FACS analysis and gene expression data support that DEX-induced GR signaling on E14.5 leads to cell cycle-phase dependent effects on RGC fate.

The regulation of cell cycle exit in progenitors is a critical factor for the construction of the cortex. Increase in the progenitor re-entry in mutant embryos with a constitutively active form (Chenn and Walsh, 2002; Chenn, 2008). Moreover, in cortices of midgestation embryos in which the endogenous levels of Axin (effector of β-catenin) were artificially increased, the number of IPCs increased due to augmented contribution from RGCs. This led to an increase in cortical thickness on E17.5 (Fang et al., 2013). Contrary, we show that E17.5 embryos exposed to DEX on E14.5, have a reduction both in cortical surface and cortical thickness. Therefore, these results taken together suggest that DEX-induced GR signaling on E14.5 has a distinct effect on proliferating RGCs, depending on whether the cells were in S-phase (BrdU+) or not (BrdU-) and overall promotes precocious neurogenesis. We hypothesized that this could occur if DEX promoted an IPC fate in non-S-RGCs (Tbr2+/BrdU-, Figure 32A) or alternatively, in S-RGCs, DEX may promote re-entry as neurogenic progenitors and subsequently leading to the generation of neurons (Tbr2-/BrdU+). The average length of the S phase and G2+M phase of RGCs at E14.5 is approximately 5 and 2.5 hours, respectively, while the cell cycle lasts around 19.5 hours (Arai et al., 2011) and Figure 32. Therefore, after 24 hours, BrdU+ labelled cells that re-entered the cell cycle, will either be in the G2M phase, have exited the cell cycle as neurons in G0 phase, or re-entered the cell cycle and are in G1 phase (Figure 33A). Therefore we decided to evaluate whether the distribution of BrdU+ cells within the cell cycle supported this hypothesis. For this purpose, we collected dorsal cortices from E14.5 embryos that were exposed to DEX and BrdU for 24 hours and processed them for FACS analysis. Indeed, DEX treatment resulted in an increase in the proportion of BrdU+ cells that were in the G0G1 phase, with a parallel reduction

in the proportion of BrdU-G0G1 cells (Figure 33B), reflecting an increase in BrdU+ RGCs that had re-entered the cell cycle (G1 phase) and committed to become neurons (G0). Also, the increased in G0G1 BrdU+, may also reflect a contribution from neurogenic S-phase IPCs that are either in G1 or have generated neurons (G0). Moreover, in DEX treated embryos there was a trend for increased proportion of BrdU+ cells in the G2M phase reflecting the neurogenic progenitors that re-entered the cell cycle (Figure 33B). Thus, we hypothesized that following DEX-induced premature GR signaling induces S-phase progenitors to adopt a neurogenic behavior, which was reflected with an accumulation of BrdU+ cells in G0G1 phase.

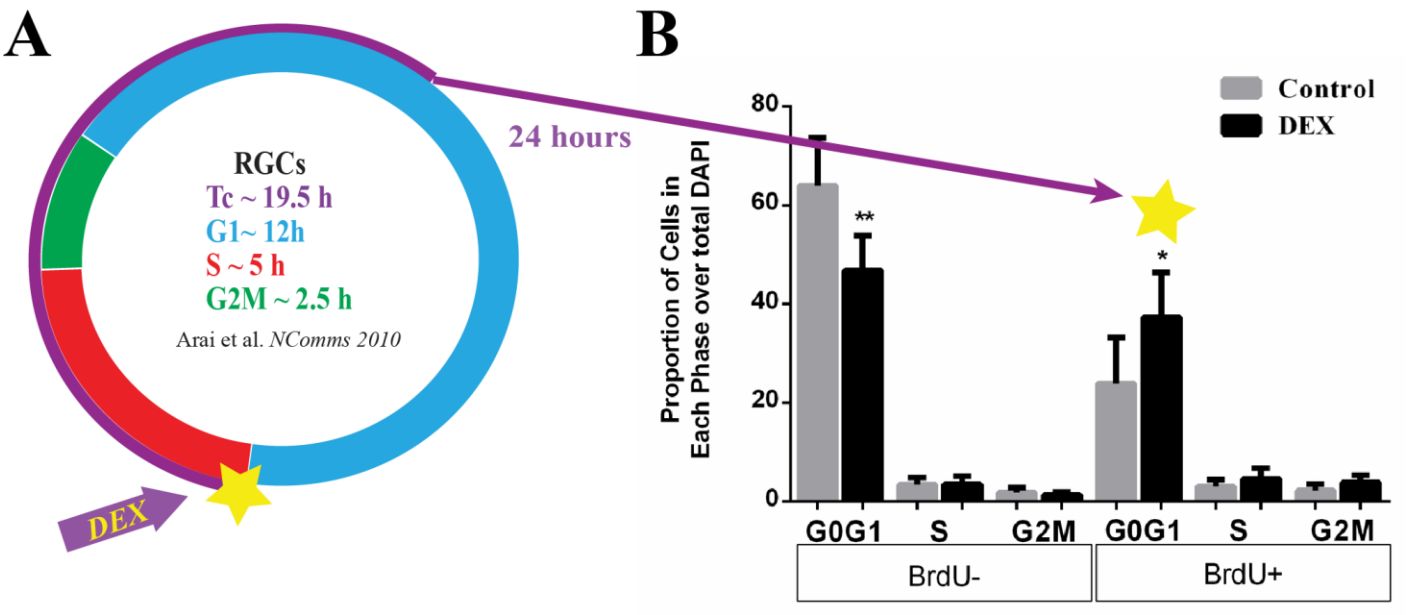
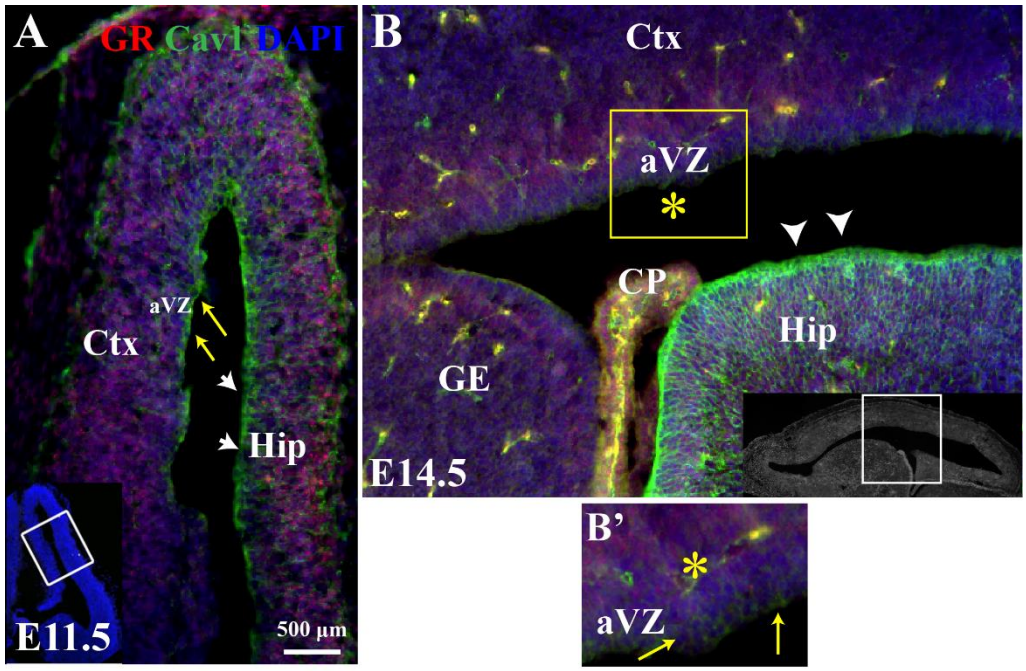


Figure 33. FACs analysis reveals a neurogenic shift in S-phase BrdU+ progenitors, after 24 hours of DEX exposure. A. S-phase RGCs exposed to DEX re-enter the cell cycle as neurogenic BrdU+-RGCs, and after **(B),** 24 hours accumulate in G1G0 reflecting an accumulation of BrdU+ neurons in G0. Proportion of (BrdU± G0G1) ± SD, Student's t-test, $n_{control}$ = 6, n_{DEX}= 7 (biological replicates); BrdU+G0G1 Control 24 ± 9.2, DEX 37 ± 9, $p < 0.05$; BrdU- G0G1: Control 64 ± 9.7, DEX 47 ± 7.1, $p < 0.01$.

3.3.9 DEX-induced premature GR signaling in RGCs promotes downregulation in c-Myc

The proliferation of neural progenitors is tightly controlled by many signaling cascades. We have shown that in cultured neural stem cells, DEX-induced GR signaling by membrane GRs in association with caveolin 1 (Cav1) and through a Src/PI3K cascade promote cell cycle exit and reduced proliferation (Peffer et al., 2014; Samarasinghe et al., 2011). Moreover, in another cell system, it was shown that membrane GR in association with Cav1 promotes activation of GSk3β, which is part of the Wnt pathway. The latter, is crucial for the regulation of neurogenesis and maintenance of the proliferation. Interestingly, the inhibition of cellular proliferation by DEX is mediated by an increase in GSK3β activity. The increased activity of GSK3β is reflected by reduced levels of downstream targets such as cyclin D1, c-Myc (Boku et al., 2008; Gulino et al., 2009; Heine and Rowitch, 2009; Nuutinen et al., 2009). Also, deletion of Gsk3β in neural stem cells, leads to aberrantly increased proliferation of RGCs with concomitant reduction in IPC production *in vivo* and upregulation of the proto-oncogene c-Myc (Kim et al., 2009; Kim and Snider, 2011) which is critical for neural stem cell self-renewal and differentiation and its functions are developmental stage specific. Specifically, *in vivo* conditional inactivation of c-Myc in neural stem cells on E15.5, leads to neuronal differentiation of the affected cells, whereas when inactivation occurs on E17.5, these cells produce glia (Nagao et al., 2008). The n-Myc gene, which belongs in the same family of transcription factors as c-Myc, was shown to be functionally complementary of c-Myc in regulating proliferation (Malynn et al., 2000). Interestingly, conditional inactivation of n-Myc in neural progenitors, causes precocious neurogenesis (Knoepfler et al., 2002). C-myc has also been known for years to be downregulated by DEX (Reed et al., 1985; Thulasi et al., 1993; Wood et al., 1994). Cav1 is highly expressed in the apical side of the neuroepithelium and colocalizes with GR in RGCs that express the receptor

in their cytoplasm both on E11.5 and E13.5 (Figures 34A, B). To evaluate the levels of c-Myc expression in the presence of DEX, we exposed E11.5 embryos to DEX for 8 hours and then the dorsal cortices were collected for analysis of c-Myc mRNA expression by qRT-PCR. At E11.5, RGCs make up 90+% of the proliferative population, whereas the numbers of neurons and IPCs are very low (Englund et al., 2005) and Figure 21. Thus, by approximation at this age we would mainly evaluate the levels of c-Myc in RGCs. Moreover, the cell cycle duration on E11.5 is ~8 hours (Takahashi et al., 1995), allowing us to evaluate c-Myc mRNA changes within the scope of an entire cell cycle. These results showed that DEX-induced GR signaling on E11.5 indeed reduces the levels of c-Myc mRNA (Figure 34C). Therefore considering the role of c-Myc in Gsk3β/Cav1/GR signaling and neurogenesis, it is tempting to assume that part of the mechanism by which DEX-induced premature GR signaling promotes precautious neurogenesis runs through downregulation of c-Myc by activation of a Cav1/Gsk3β dependent signaling cascade.



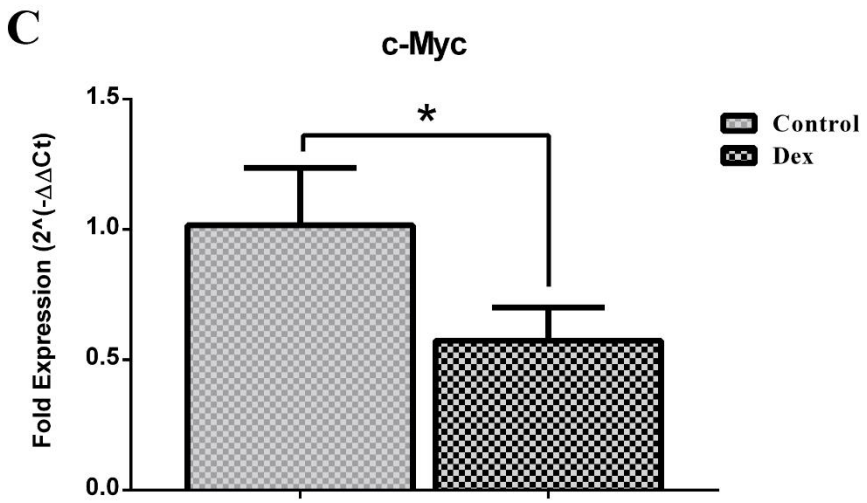


Figure 34. Premature activation of GR signaling by DEX in E11.5 NSPCs of the dorsal cortex induces downregulation of c-Myc after 8 hours. A, B. Cav1 colocalizes with GR in NSPCs of the aVZ of the E11.5 and E14.5 cortex (yellow arrows). In both ages Cav1 expressed in relatively higher levels in the hippocampus (white arrowheads). ***C.*** The expression of c-Myc, a putative target in GR-Cav1 signaling, is downregulated in E11.5 dorsal cortices exposed to DEX for 8 hours. Fold expression levels ($2^{(-\Delta\Delta Ct)}$), n=3 biological replicates (Dorsal Cortex): Control 1 ± 0.22, DEX 0.57 ± 13, $p < 0.05$.

3.4 DISCUSSION

We have previously shown that GR is expressed both in cortical RGCs and IPCs on E14.5 and that it is nuclear in the majority of the progenitors with the exception of apical RGCs that have a distinct cytoplasmic GR profile (Tsiarli et al., 2013); See Section 2.3.4). The nuclear profile of GR suggested that the receptor may have a functional role in neuronogenesis that is regulated by GC-independent mechanisms, since before E16 the brain is essentially GC-naïve (Venihaki et al., 2000). Moreover, it has become clear that GR is functional in the absence of GCs and serves as a homeostatic regulator of gene expression. Furthermore, addition of GCs disrupts this homeostatic balance and shifts the scale to GC-induced GR signaling (Ritter et al., 2012; Ritter and Mueller, 2014). In this study we show that premature activation of GR signaling by a single DEX course on E14.5, disturbs the normal process of development by favoring neuronogenesis at the expense of proliferation in RGCs. This has as a consequence, the supernumerary production in neurons of all layers, reduction in the cortical surface and thickness which is manifested overall as impaired brain growth on E17.5, 72 hours after the DEX injection. Moreover, DEX leads to permanent effects on the cortical cytoarchitecture and cognitive function of the adult animal.

3.4.1 *Cell cycle phase-dependent effects of the prematurely DEX-activated GR on RGC fate.*

It has been suggested that GR subcellular localization is intimately connected to its function in proliferating cells and furthermore it is linked to the cell cycle phase (Hsu et al., 1992; Hsu and DeFranco, 1995; Matthews et al., 2011). In GC-naïve conditions, the receptor accumulates in the cell nucleus from late G1 until the G2 phase, a slow process lasting for hours. During the M

phase, the receptor is rapidly excluded from the dividing chromosomes, whereas during early G1, GR is rapidly excluded from the nucleus (Matthews et al., 2011). GR function is modulated by baseline post-translational modifications in the form of phosphorylation that also differ depending on the kinases and phosphatases that are active during a specific cell cycle phase (Ismaili et al., 2005; Oakley and Cidlowski, 2013). Specifically, phosphorylation on Serine 211 (S211) during G1, can prime the receptor for increased responsiveness to GC-mediated signaling (Matthews et al., 2011). RGCs are the major proliferative cell type in the cortex (Pontious et al., 2008; Sun and Hevner, 2014) and contribute to neuronogenesis either directly or indirectly through the generation of IPCs. As we have previously shown, RGCs have a distinct GR expression profile (Tsiarli et al., 2013) which is in accordance to the cell cycle phase-related localization of GR as has been described by Mathews et al., (2011), whereby mitotic RGCs in the apical VZ mainly express cytoplasmic GR, whereas basal RGCs express nuclear GR. The results presented in this study also suggest that premature DEX-induced GR signaling, leads to differential effects on RGC fate depending on whether the cell was in S-phase or not during the critical window of DEX action. Specifically, after 24 hours, DEX induced an increase in the number of NSPCs that had gone through the S-phase and were positive for BrdU (28% increase over control). The increase in BrdU+ cells was localized within the RGC population, since it was reflected with an increase in the numbers of BrdU+ RGCs (Tbr2-/BrdU+; 32% increase). Overall these results suggest that premature DEX-induced GR signaling, promotes neurogenesis from RGCs either directly by forcing re-entry of S-phase RGCs as neurogenic progenitors which then divide asymmetrically to one neuron and one RGC that re-enters the progenitor zone, or indirectly by promoting the generation of IPCs from non-S phase RGCs. In other systems such as the hematopoietic system, activation of GR by DEX, promotes proliferation of erythroid

progenitors, cancer cells including neuroblastoma cells and osteoblasts (Gruver-Yates and Cidlowski, 2013; Gundisch et al., 2012; Langeveld et al., 1992; McCulloch and Tenenbaum, 1986; Panzenböck et al., 1998). Interestingly, in the case of osteoblasts, the pro-proliferative effects of the activated GR are actually directed to a cycling cell population that is primed to differentiate (McCulloch and Tenenbaum, 1986), hence GR signaling promotes differentiation.

DEX activation of GR promotes G1 arrest in cycling neural progenitors *in vitro*, by stimulating p53 nuclear translocation and transcription of p21**,** nonetheless, without promoting apoptosis (Crochemore et al., 2002)**.** It is possible that one likely mechanism by which DEX-induced GR signaling ultimately promotes neurogenesis in S-phase RGCs (Tbr2-/BrdU+), is by initiating a similar signaling cascade as the one reported by Crochemore et al., (2002). Specifically, DEX-induced GR signaling may indirectly, promote activation of the p53/p21-dependent cell cycle control, via inhibition of BRCA1 expression. Is its known, that the unliganded GR, positively regulates the expression of BRCA1 by direct association with the BRCA1 promoter. Interestingly this association is repressed following 24 hour exposure to GCs (Ritter et al., 2012; Ritter and Mueller, 2014), thus providing a reasonable hypothesis on how DEX may be acting in NSPCs. BRCA1 is essential for cortical development (Pao et al., 2014; Pulvers and Huttner, 2009). Loss of BRCA1 results in nuclear translocation of p53 and apoptosis *in vivo*, whereas neural progenitors in culture with half dose of BRCA1 continue to proliferate (Pao et al., 2014). The axis GR-BRCA1/p53-p21 is likely to be the mechanism by which DEX promotes neurogenic divisions in S-phase RGCs, since it goes along with the above observations that a G1 arrest by DEX primes neural progenitors for differentiation (Crochemore et al., 2002). Accordingly, in the 24 hour DEX-treated embryos a greater number of S-RGCs re-entering the cell cycle and at the same time a concomitant increase in neurons produced by S-phase

progenitors (Tbr2-/BrdU+), suggesting that S-phase RGCs where primed by DEX to re-enter the cell cycle as neurogenic progenitors. Moreover, due to the interkinetic nuclear migration that RGCs undergo, S-phase RGCs are located in the basal aspect of the VZ. BRCA1 is most highly expressed in the cortical neuroepithelium during the peak of proliferation (~E14.5). Additionally, its expression is higher in the basal parts of the progenitor zone (Pao et al., 2014) and thus, reminiscent of the high nuclear GR expression in the same zone (Tsiarli et al., 2013), see section 2.3.3). Thus the spatial localization of GR and BRCA1 would be in agreement along with the existence of a GR-BRCA1/p53-p21 axis in these cells. Moreover, there has been a number of results associating different DEX-binding to GR according to the cell cycle phase and GR concentration itself. Specifically, it was shown that during S-phase the proportion of DEX-GR in the nucleus is higher both in proliferating mouse and human lymphoid cells (Distelhorst et al., 1984). Moreover in HELA cells, the concentration of GR increases during entry in the S phase (Cidlowski and Cidlowski, 1982). The same phenomenon seems likely to apply in S-phase RGCs. S-phase RGCs are located in the basal VZ and have high levels of nuclear GR relative to their apical counterparts, putatively making them more vulnerable to DEX.

Membrane-bound GR in association with caveolin 1 (Cav1), can also mediate rapid DEX effects on cell cycle regulation (Matthews et al., 2008a; Peffer et al., 2014; Samarasinghe et al., 2011). Specifically, in the lung epithelial cell line A549, DEX promotes phosphorylation of Cav1, and protein kinase B (PKB)/Akt via the src kinase and leads to growth arrest and accumulation in G1/S (Matthews et al., 2008a). This pathway is also functional in neural stem cells *in vitro* and regulates their proliferation (Peffer et al., 2014; Samarasinghe et al., 2011). Moreover, in the A549 cells, Cav1-dependent rapid GR signaling, induced activation of glycogen synthase kinase-3β (Gsk-3β), and of the mammalian target of rapamycin (mTOR).

Interestingly, Cav1 levels are higher in the apical side of the VZ (Fig.3.16 A and B), coinciding with the zone of enhanced cytoplasmic GR expression on E13.5 (Tsiarli et al., 2013), Section 2.3.3). RGCs on the apical VZ are either in mitosis, in late G2 or early G1, but not in S-phase. Therefore, in this sub-population of RGCs (non-S phase RGCs), activation of GR by DEX may promote GSk3β activation and subsequently, changes in Wnt signaling that promote production of IPCs cells from RGCs at the expense of their self-renewal (i.e., re-entry in the cell cycle as neurogenic progenitors that give rise to IPCs, and not as proliferative RGCs). GSK3β is a serine/threonine kinase, that is critical for the homeostasis of NPC function during cortical development (Kim and Snider, 2011). Specifically, GSK3β manages the exquisite balance between proliferation versus differentiation of NSPCs by being a principal regulatory component of the canonical Wnt/β-catenin pathway. The Wnt/β-catenin pathway is one of the major morphogenetic signals that promotes proliferation of NSPCs during development of the brain. The major effector of the pathway is β-catenin, which functions as a transcriptional inducer of cell cycle related genes such as Cyclin D1 and c-Myc (He et al., 1998). GSK3β phosphorylates not-activated β-catenin and tags it for degradation by the proteasome. Interestingly, high GSK-3β signaling has been proposed to promote asymmetric divisions of RGCs and the generation of IPCs and neurons (Hur and Zhou, 2010). Moreover, upregulation of axin, a regulator of the canonical Wnt pathway, which can also physically associate with GSK-3β (Kim et al., 2009; Kim and Snider, 2011) to promote β-catenin degradation, stimulates asymmetric divisions of RGCs to IPCs causing an increase in IPC number (Mao et al., 2009). Consistent with this reasoning, DEX promotes adipocyte differentiation in a GR-dependent manner, by upregulation of Axin 2 (Naito et al., 2013). Therefore, it is possible that in non-S-phase RGCs DEX promotes IPC production partially through rapid GR signaling and activation of GSK-3β signaling

cascades. Also, another putative player in the DEX-induced IPC generation from non-S-phase RGCs, might be mTOR, which was also shown to be a downstream target of rapid GR signaling (Matthews et al., 2008b). RGCs express Pax6, which is a critical regulator of neurogenesis. Pax6 also regulates the transcriptional activation of Tbr2 and promotes the generation of IPCs (Englund et al., 2005; Fishell and Kriegstein, 2003; Kohwi and Doe, 2013). It has been shown that Tbr2 regulation by Pax6, occurs in a cell-autonomous manner and it is dependent on the activation of the mTOR complex 1 (mTORC1) pathway (Díaz-Alonso et al., 2014). Moreover, increased mTOR signaling promotes precocious differentiation of cortical progenitors at the expense of their proliferation (Magri et al., 2011).

Another possible mechanism by which DEX-induced GR signaling may promote neurogenic divisions of RGCs either to neurons or IPCs is by altering the kinetics of the cell cycle. It is well established that the length of the G1 phase cycle in neurogenic progenitors is increased compared to proliferating progenitors, with the highest G1 length being of neurogenic IPCs at 21.3 hours. Neurogenic RGCs on the other hand have a G1 that lasts 11.7 hours (Arai et al., 2011). Moreover, neurogenic progenitors have a shorter S-phase compared to proliferating progenitors. Baghdassarian et al. (1998) showed in phytohemagglutinin-stimulated human lymphocytes, DEX causes as a primary response a lengthening in G1 phase with a parallel reduction in S-phase. After 24 hours S-phase is increased in length. Part of the mechanism by which DEX modulates G1 and S length is by promoting an increase in p27 levels. It is tempting to hypothesize that a similar mechanism involving p27 may be also taking place in DEX-exposed RGCs, and inducing a fate change from proliferating to neurogenic progenitors. The role of p27 in promoting cell cycle exit and increase in G1 is well studied (Caviness et al., 2009; Nguyen et al., 2006; Tarui et al., 2005) and it has been suggested that p27 interacts with the Notch pathway

to regulate the progressive increase in G1 length during the normal course of neurogenesis (Caviness et al., 2009).

3.4.2 *Increased number of neurons in a smaller cortex*

3.4.2.1 DEX favors a neurogenic fate in RGCs, but with a price

This study has shown that a single course of DEX at a clinically relevant dose on E14.5, leads to an impairment in brain growth, which is manifested as reduced cortical surface and reduced cortical thickness on E17.5. Nevertheless, despite the reduction in cortical thickness, these animals have increased neuronal numbers corresponding to an increase in all cortical layers as seen with specific laminar markers. However, we cannot preclude that this increase is actually an increase in packing density rather than an increase in neuronal number *per se*, since precocious differentiation would eventually lead to a smaller overall cortex. Along these lines, a depletion of the progenitor pool in the form of asymmetric divisions, would also favor neuronogenesis at the expense of gliogenesis, which starts around E16 (Rowitch and Kriegstein, 2010). Preliminary results from this study, in embryos exposed to a single course of DEX on E14.5 and collected on P22, indicate that these animals show alterations in the white matter and specifically in the corpus callosum. Impairments in myelination as a result of antenatal GC exposure have been shown in multiple studies (Braun et al., 2013).

Furthermore, changes in cortical thickness and surface, are directly linked to the RGC progenitor pool and their ability to shift from a mainly symmetric self-renewal state to asymmetric neurogenic divisions that give rise to IPCs or neurons. Accordingly, deletion of FGF genes (R1-3), that control this developmental fate shift, results primarily in an increase in RGC-born IPC, with a concurrent depletion in RGCs which ultimately leads to a reduction in cortical

thickness and surface (Kang et al., 2009). Moreover, Pilaz et al. (2009) demonstrated that an induced reduction – rather than increase- in the length of G1 by overexpression of cyclin D1 or E1 on E15, leads to an initial increase in the self-proliferation of RGCs only to switch to increased production of IPCs after 24 hours. Our FACs analysis showed that DEX exposure for 24 hours resulted in an decrease in the proportion of BrdU- cells that are in G1, whereas, the proportion of BrdU+ cells in G1 was increased (Section 3). This differential DEX effect depending on whether the cells had gone through the S-phase (BrdU+) or not (BrdU-), again suggests differential effects of DEX-induced GR signaling in RGCs depending on whether the cells were in S-phase.

Since the increase in Tbr2+ IPC cells was driven by RGs that were not in S-phase during the DEX-induced GR signaling, it is tempting to assume that a similar signaling events such as the one described by Pilaz may be induced in non-S-phase RGCs. Moreover, Pilaz and colleagues (2009) showed that even if G1 is altered in progenitors, they continue to produce the right neuronal progeny for the respective developmental stage but, at the expense of the previous neuronal layer which was "skipped" due to the G1 increase. Specifically, the progenitors mainly produce LII/III on E15 and LIV production is reduced. Our results show that DEX induces an increase in neurons of all layers, further supporting a dual effect on RGCs, rather than promoting the generation of one layer at the expense of another. This result can be better explained if we hypothesize that the activated GR concomitantly induces the production of two neuronal waves: first it promotes asymmetric neurogenic divisions and the production of neurons primarily for deep layer V and at a less extent, deep layer VI, which are normally being born at E14.5 (Takahashi et al., 1999). Concomitantly, GR promotes the production of upper layer II-IV

neurons either from S-phase RGCs or indirectly by the generation of IPCs from non-S phase RGCs (indirect neurogenesis).

The distinct cytoarchitectural effects of DEX we observe on E17.5 are reminiscent of the cortical cytoarchitecture phenotype in reelin heterozygote mice, in which the thickness of the cortex is reduced albeit the neuronal *packing density* is increased. This is the end-result of a reduction in reelin-expressing cells in the adult brain, putatively of GAD67 interneurons which leads to a hypoplasia of the pyramidal neuron spines (Liu et al., 2001). Interestingly, postnatal (P) DEX exposure of P3 mice pups, increases the proportion of interneurons in the cortex with a parallel decrease in cortical thickness (Baud et al., 2005). Even though this study used postnatal mice, it indicates that DEX may have an effect on interneuron genesis and biology.

Although we have not explored the effects of premature DEX-induced GR activation on interneuron genesis, we have shown that GR is highly expressed in interneuron progenitors (Sox2+) and mature interneurons of both of the medial and lateral ganglionic eminence (MGE and LGE), the former being a major source of cortical interneurons (Batista-Brito and Fishell, 2009). GR expression in the apical progenitors of the GE, which also undergo INM (Pilaz et al., 2009) is similar to the apical progenitors of the cortex where a strong cytoplasmic GR profile is observed (Tsiarli et al., 2013). Therefore, premature activation of GR signaling in interneuron progenitors may affect their proliferation, migration and integration in the cortex, including their reelin production and contribute to the changes we observed in cortical cytoarchitercture. Interestingly, Tbr2+ progenitors were shown to have a role in attracting and guiding subpallial interneurons to migrate and integrate in the cortex. Deletion of the Tbr2+ cells results in reduced numbers of interneurons in the cortex on E18.5 (Sessa et al., 2010). Thus another mechanism by which DEX indirectly may have affected the dynamics of interneurons is through the increase in

Tbr2+ cells which could attract more cortical interneurons and thus contribute to the increased number of DAPI+ cells we observed in the cortical plate.

Interestingly, increased glucocorticoid levels have been shown to have differential effects on brain reelin expression depending on the age and the sex of the animal. Specifically, exposure to repeated stress during the sensitive neonatal period, permanently increases the levels of hippocampal reelin expression in male mouse pups but not in females (Gross et al., 2012). Contrary, repeated exposure of adult rats to corticosterone over a span of 21 days but not to restraint stress, reduces the number of reelin immunoreactive cells in the hippocampus and promotes the development of depressive symptoms (Lussier et al., 2011). The hippocampus is densely populated with GRs, and as we have shown ((Tsiarli et al., 2013), Section 2) this applies from the very early stages of hippocampal morphogenesis, thus making it particularly vulnerable to the effects of GCs. The aforementioned studies, highlight the different effects of GR signaling on reelin signaling cascades, depending on the developmental stage.

Beyond its traditional role in neuronal migration, reelin has a very important role in neurogenesis which only recently has started to draw attention. Specifically, reelin is secreted by the Cajal Retzius cells, a transient cell population that resides in the marginal zone during the embryonic life and is critical for instructing the migration of the nascent neurons to their respective position in the cortical plate (Gil-Sanz et al., 2013). The onset of neurogenesis coincides with the appearance of the Cajal Retzius cells and the initiation of reelin production. Also, the switch of symmetric to asymmetric neurogenic divisions in RGCs in the progenitor zone, is tightly under the control of the Notch pathway (Fishell and Kriegstein, 2003). Reelin induces activation of the Notch pathway in RGCs and promotes the shift in the asymmetric divisions of RGCS and thereby controlling neuronogenesis (Lakoma et al., 2011). Reelin gain-

of-function in neural progenitors under the nestin-driver *in vivo*, induces upregulation of Notch target genes, NICD and Hes1, which is accompanied with increased IPC (Tbr2+) numbers, increased cell cycle exit and neuronogenesis. The effect of reelin in acting upstream and enhancing Notch signaling in RGCs and thereby regulating their fate, is independent on its function on postmitotic neuron migration (Lakoma et al., 2011). As we have showed, GR expression is highest in Cajal Retjius cells and specifically it is intensely nuclear (Tsiarli et al., 2013, see section 2.3.2.2).

Considering the connection of glucocorticoids with reelin expression in the postnatal brain, we can speculate that premature activation of GR signaling by DEX promotes upregulation of reelin, which in turn acts on RGCs and enhances Notch signaling to promote asymmetric divisions and precocious neurogenesis resulting then in increased neuronal numbers and a smaller cortical plate. Interestingly, corticosterone induces upregulation of reelin expression in osteoblasts (Ma et al., 2012). Moreover it is important to note that reelin has been in the spotlight in relation to the pathogenesis of neuropsychiatric disorders. Considering that increased GC levels during pregnancy have been also linked to the pathogenesis of neuropsychiatric and affective disorders, the role of GR in affecting reeling signaling during development, is definitely an association needed to be explored in depth.

3.4.3 *Relation to previous studies on prenatal GC effects on the developing brain.*

GCs effects on the developing brain have been the topic of many studies, especially following their introduction in obstetrics for the management of preterm labor. In agreement with the results from most studies, we show that a single injection of a clinically relevant dose of DEX leads to a reduction in brain growth in terms of reduced brain to body weight ratio and also

reduced cortical surface and thickness. Interestingly, in our model, body weight was slightly and significantly increased, but still this increase in body weight was not followed by a comparable trend in brain weight (Section 3). This apparently discordant effect of DEX on body weight can be explained by the fact that many of the studies that have shown a reduction in birth weight used either higher doses of DEX and/or used multiple courses of DEX and at different gestational stages (Noorlander et al., 2006; Noorlander et al., 2008; Noorlander et al., 2014; Zuloaga et al., 2011), whereas we explored the effects of a single course of DEX at midgestation. In our case, a single course of DEX in a clinically relevant dose allows us to dissect the "baseline" effects of premature DEX-induced GR signaling, without the confounding effects of multiple injections or of higher and non-clinically relevant DEX doses. Along similar lines, our preliminary results of caspase-3 staining, did not reveal gross differences in apoptosis in the E17.5 cortex, following DEX exposure on E14.5. Contrary many studies have shown that prenatal DEX exposure leads to apoptosis (Sundberg et al., 2006; Zuloaga et al., 2011) which in turn may result in a smaller brain postnatally, with especially high impact on the hippocampus (Khozhai and Otellin, 2008; Uno et al., 1990; Uno et al., 1994). Again these studies have used multiple injections of DEX and/ or non-clinically relevant doses which would be toxic for the progenitors

One unique feature of our experimental design is that the embryos are exposed to DEX before the endogenous GC-production begins i.e., before E16. Similarly in the human condition, DEX is administered between 24 -34 weeks of gestation for the management of preterm labor (WHO). During this age the brain undergoes a dramatic development shift which is mostly evident in terms of cortical surface expansion and concomitant elaboration of the cortical gyrification pattern (Kapellou et al., 2006). Additionally, while for the majority of gestation the

circulating GC levels in the embryo were low, due to the protective role of the placenta, they steadily increase close to term promoting organ maturation and priming the fetus for labor (Braun et al., 2013; Ishimoto and Jaffe, 2011). Therefore two important points need to be noted about this condition; first during the 24-34 week time-window that DEX is likely to be used, endogenous levels of GCs are low and secondly, this is a very sensitive period for brain development with highly dynamic changes in cortical development taking place. Our experimental design allows us to mimic the low-GC human embryonic brain environment while also, being as close as possible in terms of neurodevelopmental events.

A series of studies from Noorlander and colleagues (2008, 2014) used the same DEX dose in mice, but instead chose E15.5 as the exposure time and examined the effects on hippocampal development at different pre- and post-natal stages. Interestingly, DEX lead to an increase in activated caspase 3 positive cells in the hippocampus and reduction of proliferation in terms of Ki67+ cell numbers. Moreover, DEX caused long-lasting effects on cortical function manifested in terms of impaired spatial learning and memory (Noorlander et al., 2008). At this point we have to note that in our study we used pure DEX (MW 392.4 g), rather than the clinically used DEX phosphate (MW 516.4 g) which contains sulfites as preservatives and was used by Noorlander and colleagues (2008, 2014). The advantage of using pure DEX over the phosphate form, is that it allows us to observe direct effects of prematurely induced GR signaling, without the intermediate steps that would intervene until the phosphorylated form hydrolyzes to bioactive DEX (Miyabo et al., 1981). Also, it has been suggested that the sulfites contained in the clinical DEX form may be actually causing neurotoxic effects in the developing brain (Baud et al., 2001).

In addition our study was centered on the specific effects of premature DEX-induced GR activation on NSPC biology during the peak of neuronogenesis (E14.5, midgestation). Specifically we focused on the two major types of cortical progenitors the RGCs and IPCs, which in our knowledge is the first attempt to dissect the role of GR and its premature GC-induced signaling on neurogenesis and distinct types of NSPCs *in vivo*. Also, we show that a single course of DEX results in an increase of cells going through S-phase as reflected with an increase in BrdU+ cells after 24 and after 72 hours, rather than reducing them as previous studies have shown (Sundberg et al., 2006). Interestingly, prenatal betamethasone (the second most common sGC used for the management of preterm labor) administered in rat embryos and at a clinical relevant dose, can lead to an increase in proliferation postnatally as seen by increased [3H]thymidine incorporation and without causing apoptosis (Scheepens et al., 2003).

3.4.4 *Homeostatic role for non-GC bound GR in neurogenesis?*

In many of the studies examining the effects of GCs on different organ systems and clinical settings, the role the GR beyond mediating the effects of GCs has largely been ignored. GR is a transcription factor and may be independently acting in the absence of its traditional GC ligands to modify target gene expression. Recently the role of unliganded GR in breast cancer has been explored. In wild type mammary cells, the unliganded GR positively and directly regulates BRCA1 expression by direct association on the BRCA1 promoter and in collaboration with GABP-β (Ritter et al., 2012; Ritter and Mueller, 2014). This positive regulation is disrupted when hydrocortisone is present, thus providing a foundation for the role of stress in BRCA1 down-regulation seen in sporadic breast cancer (Ritter and Mueller, 2014). Furthermore, the same group recently showed that the role of unliganded GR in controlling gene expression

expands beyond BRCA1, to many other genes including genes that are critical in organogenesis such as Cyclin D2 (Ritter and Mueller, 2014). Importantly, cyclin D2 is implicated in the generation of IPCs and it is also expressed in the human SVZ (Glickstein et al., 2009). Interestingly, these studies have sparked another line of research focusing on GR as diagnostic factor in breast cancer. Vilasco et al. (2013), showed that BRCA1 levels are intimately linked with the levels of GR and its basal phosphorylation on S211 which is critical for "maximal activation of GC signaling (Chen et al., 2008; Matthews et al., 2011). Silencing BRCA1 expression by siRNA, reduced both basal and DEX- induced levels of GR-S211. Within the context of the developing brain, we have shown that GR is highly expressed in the developing telencephalon and cortical neuroepithelium and it is mainly nuclear. Furthermore GR is expressed in a gradient along the rostro-caudal axis with higher expression levels in the caudal telencephalon and specifically in the hippocampus (Tsiarli et al., 2013). Moreover, DEX-induced GR-signaling can affect gene expression from an early age i.e., c-Myc on E11.5 (see Figure 34C), making the hypothesis for a homeostatic role for the unliganded GR in neurogenesis very likely.

The role of prenatal stress on the offspring is a topic that has been in the center of attention for many years now. Increased levels of GCs as a result of maternal stress can lead to permanent changes in the physiology of the offspring that impact metabolic and cardiovascular aspects but also cognitive function through a premature activation of GR and reprogramming in the basal functioning state of the HPA axis (Harris and Seckl, 2011; Reynolds and Seckl, 2012; Speirs et al., 2004). Importantly, with the advent of the genomics, changes in gene expression resulting from an adverse uterine environment during pregnancy, including increased GC levels, have been characterized as risk factors associated with the development of neuropsychiatric and

affective disorders (Huang, 2011; Lupien et al., 2009). Many of these genes or their products are linked to GR signaling such as Reelin and GABA, FKBP5 (Binder, 2009; Lussier et al., 2011; McEwen, 2005).

The prefrontal cortex and the hippocampus two regions that are highly linked with the development of neuropsychiatric and affective disorders are highly enriched in GRs and the causative link between abnormal GR function and a risk for developing neuropsychiatric and affective disorders, has been shown in many clinical and non-clinical studies (McEwen, 2005; McGowan et al., 2009; Welberg and Seckl, 2001). Interestingly, examination of cortical thickness by MRI in school aged children, that were prenatally exposed to GCs (mean gestational age 28 weeks) but born at term, showed that there is a bilateral thinning of the cortex. The area mostly affected was the anterior cingulate cortex which is densely populated with GRs. Furthermore, cortical thinning in these children was associated with the development of affective problems (Davis et al., 2013). These results point to a necessity for a better examination of GRs role in the developing brain, by looking at it through the prism of signal transduction of a dynamic transcription factor, rather under the strict line of GR being a mediator of GC effects. Obtaining a more clear understanding of the role of GR in the developing brain and its putative homeostatic/regulator role in neurogenesis in association with BRAC1, reelin or other molecules will allow a better understanding of the effects of GCs on the developing brain. Also, it would improve current GC treatments or lead to the development of new treatments characterized by more health benefits and reduced side-effects on neural development.

4.0 GENERAL DISCUSSION

The introduction of sGCs in medicine for the management of preterm labor, has had a dramatic effect on neonatal morbidity and mortality. Undoubtedly, many neonates have been saved due to the meticulous and inspiring work of Dr. Liggins which lead to the discovery of the benefits of GCs in improving survival of preterm babies and reducing neonatal mortality due to the various complications of preterm labor. Nevertheless, no medicine is a panacea itself and as it is often the case potential side-effects are set aside in the light of major benefits. *In utero* exposure to GCs and especially sGCs that are resistant to placental degradation, will globally affect the developing embryonic organ systems, amongst them the brain. As we have shown, GR, the mediator of the intracellular effects of GCs, is expressed in the mouse embryo brain from very early stages of neurogenesis and moreover, it can affect gene transcription and alter NSPC properties when it binds DEX (Figure 3.16). Two major facts corroborate the hypothesis that the unliganded GR may be acting independently of GCs during this period to regulate neurogenesis: First, GR is expressed in high levels in the nucleus of the majority of progenitors and neurons of the neuroepithelium, but at the same time in apical progenitors it has a restricted cytoplasmic localization seen throughout the apical surface of the telencephalon. Secondly, binding of GR to DEX before the natural onset of GC production by the embryo (E16), results in changes in the proliferation of RGs and leads to impairment of cortical growth perinatally (this study) and behavioral changes that last in adulthood (this study, (Noorlander et al., 2008; Noorlander et al.,

2014)). We do not refer to DEX binding to GR as activation since the unliganded GR has been clearly shown to have an active role in gene expression and maintaining homeostasis in the cell (Ritter et al., 2012; Ritter and Mueller, 2014; Vilasco et al., 2013). These observations highlight the fact that the function of GR should be re-evaluated, but not in a GC-centralized scheme, but rather as a dynamic transcription factor which is at the nexus of critical developmental signaling cascades. Its function is dynamically regulated by the cell cycle phase and at the same time, the GR itself regulates cell cycle (Ritter et al., 2012; Ritter and Mueller, 2014; Vilasco et al., 2013) ultimately affecting organogenesis and development. In many studies on the effects of increased GC levels *in utero* either as a result of stress, traumatic experience, and disease or due to exogenous treatment, the hippocampus and the prefrontal cortex have played the leading roles due to their tight association with the pathophysiology of neuropsychiatric and affective disorders and due to their high density of GRs. The expression levels of the GR dictate the amplitude of the GC response. Furthermore another aspect that is not frequently discussed, is that following a GC-induced GR signaling, the levels of the receptor are downregulated due to negative feedback. This mechanism has been associated with the pathogenesis of depression (Harris and Seckl, 2011; Ridder et al., 2005; Webster et al., 2002) and has been shown in victims of suicide with a background of childhood abuse (McGowan et al., 2009). Furthermore, exposure to DEX during the first week of life in mice, results in a permanent and specific downregulation of GR (Felszeghy et al., 1996), suggesting that side-effects of premature GC-GR binding *in utero* may not only be a result of altered signaling but also due to a permanent downregulation of the receptor in the affected areas. This hypothesis would especially affect those developing brain areas that are more susceptible to GCs due to their high density of GRs, such as the cortical

progenitor domain and the hippocampus (this study), as has been shown for neurogenesis in the adult hippocampus (Fitzsimons et al., 2013).

GC actions can vary depending on the cell and tissue type and the age. Also, mouse models with genetically modified GR expression may differ in phenotypes and behavior despite seemingly having the same effect on GR levels or signaling. This is attributed to the pronounced diversity of GR transcripts and isoforms which is a result of alternative splicing and/or posttranslational modification of the receptor protein (Oakley and Cidlowski, 2013). In the human cortex, GR variants are expressed in a dynamic pattern during critical periods of neuroplasticity and development of the pyramidal and astroglial components, reflecting vulnerability of the developing cortex to excessive levels of GCs (Sinclair et al., 2011). In these study, high nuclear localization of the main GR variant (GRα), was observed in the pyramidal neurons of the maturing frontal cortex suggesting a role for these cells in the stress response and is reminiscent of the high nuclear GR levels we observed in LV. Moreover during the first trimester of life, a transcriptionally hyporesponsive GR is prevalent, in agreement with the existence of a stress hyporesponsive period during the neonatal life. Therefore, considering this high degree of heterogeneity in GR isoforms, one must be careful on how to interpret results of GC action in the system of interest. Therefore it is tempting to speculate that such variability in GR variants may exist in the developing cortex niche and in the different progenitor types, allowing differential regulation of neurogenesis in a cell-type dependent manner.

In the light of accumulating data for a dynamic role of the unliganded GR in gene expression and cellular homeostasis, in combination to the results on the effects of premature GR “activation” on neurogenesis and cortical development contributed by the current study, the role of the unliganded receptor in these processes should be further explored, through a non-GC-centralized scheme. Having this knowledge would allow the improvement of current treatments not only for preterm labor management but also other clinical settings such as cancer and depression.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

5.1.1 CONCLUSIONS

This study was fuelled by the combined fact that antenatal use of GCs can lead to detrimental effects on neurogenesis, cortical development and the offspring behavior and yet, the exact role of the GR in neural progenitors and in neurogenesis was not explored beyond its traditional view as a mediator of GC signaling. Nevertheless, it has become clear that GR is a dynamic transcription factor which can regulate, highly important developmental genes such as BRCA1, in the absence of GCs. Importantly in order for GR to maintain this regulatory profile, GCs should be absent. Therefore it is clear, that GR is emerging through this studies as a homeostatic regulator and a *bona fide* transcription factor lying in the nexus of many signaling cascades and which does not require GCs to function. Rather GCs, shift the regulatory profile of the receptor and alter its dynamic range of action. The results from my study corroborate for a homeostatic role of the unliganded GR in neurogenesis and cortical development which is tightly linked to the cell cycle phase. The homeostatic role of GR is disrupted by DEX and leads to precocious neurogenesis by altering neuronal progenitor properties depending on whether the progenitor was in S-phase or not. The line of data that support this notion are:

1. GR protein is highly expressed in neural progenitors from early neurogenesis i.e., E11.5
2. It is predominantly nuclear regardless of the fact that the embryonic brain is GC-naïve before E16.
3. Premature DEX-induced activation of GR on E11.5 causes downregulation of c-Myc supporting that GR is functional and responsive to GCs and premature activation of its signaling may disrupt its homeostatic role in neurogenesis.
4. GR protein is differentially expressed in RGCs with apical RGCs expressing predominantly cytoplasmic GR, whereas basal RGCs express predominantly high levels of nuclear GR. This distribution is in agreement with a high nuclear expression of GR in interphase cells and especially in S-phase cells representing basal RGCs, and cytoplasmic expression in M phase cells, representing RGCs on the apical surface of the VZ. This distribution is in agreement with a cell cycle-dependent distribution and function of GR in the absence of GCs (Matthews et al., 2011).
5. Accordingly, premature DEX-induced activation of GR signaling on E14.5 (GC-naïve conditions), promotes precocious neurogenesis by differentially affecting progenitor fate according to the cell cycle phase. In non-S-phase RGCs, premature GR activation induced indirect neurogenesis by promoting the IPC fate (28% increase in IPC numbers), whereas in S-phase RGCs, DEX promoted a competence shift and cell-cycle re-entry as neurogenic progenitors (direct neurogenesis). This was reflected as a 32% increase in the number of S-phase RGCs in the VZ reflecting a mixed population of cycling neurogenic RGCs and their neuronal progeny and a 31% increase in the number of neurons in the IZ. Moreover, DEX may have promoted/accelerated neurogenesis from IPCs, which could also have contributed in the increase in neurons of the IZ. This supports the notion that

premature DEX-induced GR signaling, disrupts a homeostatic role of the unliganded GR primarily in RGCs, and leads to precocious neurogenesis, either directly through S-phase RGCs or indirectly through the generation of IPCs from non-S phase RGCs.

6. Disturbance of the homeostatic role of GR by DEX in E14.5 progenitors, lead to precocious neurogenesis, by promoting a neurogenic fate in progenitors, increase in the total neuronal output that populated the cortex, but in a smaller cortex both in the radial and lateral dimension. These results are in agreement with a depletion of the progenitor pool, by favoring a neurogenic fate when GR signaling is prematurely activated by DEX. Two points that support this conclusion: Studies showing an expansion of the RGC pool show that the cortex also expands at the lateral dimension (Chenn and Walsh, 2002). Furthermore, if the IPC population itself is expanded in the presence of DEX, we would expect an increase in the radial extent of the cortex as shown in many studies (Kim et al., 2009; Pontious et al., 2008; Sahara and O'Leary, 2009). In addition, S-phase progenitors (BrdU+), generated a higher proportion of neuronal output (BrdU+) that occupied the cortical plate on E17.5 but a smaller proportion of these cells remained as progenitors in the progenitor zone on E17.5 suggesting that the self-renewing pool of progenitors was depleted in the presence of DEX because they acquired a neurogenic fate. This was also reflected as an increase in neurons of all layers including deep layer VI. If re-entry of both RGCs and IPCs had increased we would expect an increase mostly in deep layers but also an expansion in the cortical plate size as seen by Mairet-Coello et al. (2012). Nevertheless, in our study, despite the increase in neuronal number, the cortical size was smaller both in the radial and lateral dimension, supporting the notion that premature GR

activation of GR by DEX, favored a neurogenic fate in the progenitors and lead to a depletion of the progenitor pool.

Collectively, my data suggest that the unliganded GR, has a regulatory role in the proliferation of neural progenitors and especially of RGCs. GRs' regulatory role in RGCs is primarily highlighted by its' distinct expression profile in apical versus basal RGCs, which is in accordance with a cell cycle phase-specific localization of GR and with the INM behavior of RGCs. Furthermore, the homeostatic role of unliganded GR in RGCs, is further supported by the fact that, premature DEX-induced GR signaling has differential effects on RGC behavior depending on the cell cycle-phase of the cells. This in turn reflects a differential function and responsiveness of the unliganded GR to GCs depending on the cell cycle phase of the progenitor. In addition, the results from this study are in accordance to previous published data from different cell lines (McCulloch and Tenenbaum, 1986), DEX-induced GR activation primed the progenitors to a neurogenic fate i.e., differentiation.

Therefore my results support the view of the unliganded GR as a dynamic transcription factor which regulates cellular proliferation, neurogenesis and development and thus highlighting the need to decouple the view that GR functions solely through GCs. Rather, it should be considered that GR competence to activate and regulate cellular and developmental processes is intertwined with the demands of the cellular machine in response to intrinsic and extrinsic factors. GC activation is a switch to shift the competence of the GR towards maintaining the homeostasis of the cell in response to the changing environment. Adopting this view would allow a better understanding on the actions of GCs in different health and disease contexts. Also it can benefit

the improvement of current treatments not only for preterm labor management and avoid potential neurological side-effects in the neonates, which was the rationale behind this study, but also improve the use of GCs in other clinical settings such as cancer and depression.

5.1.2 FUTURE DIRECTIONS

5.1.2.1 *The effects of premature GR activation by DEX on E14.5 are permanent and are manifested as changes in cortical cytoarchitecture and in cognitive and affective behavior in the postnatal animal.*

One of the main questions one may have from the above results, is whether the effects of premature DEX-induced GR activation on neurogenesis leads to permanent changes in the offspring. To evaluate this possibility we conducted pilot studies that examine the cortical cytoarchitecture and behavior of animals that were exposed to DEX on E14.5 and used for analyses at different ages. Pilot anatomical data derived from adolescent animals (Postnatal day, P22 group) exposed to DEX on E14.5, indicate that the depth of the corpus callosum is increased perhaps as a consequence of the increased number of neurons in the cortex that might contribute in white matter density. Alternatively, a change in the competency of the NSPCs at E14.5 5 might have affected the progenitor pool that would later give rise to glia.[7].

Interestingly, the formation of the corpus callosum is under the tight control of Satb2 and Ctip2 (Srinivasan et al., 2012). Cells of both types are increased following DEX exposure (See section 3.3.3, Figure 23B) and also, GR is specifically and highly expressed in layer V Ctip2+ neurons (See Section 2.3.2.6, Figure 15), suggesting that actions of DEX-induced GR signaling may not be limited in the progenitor stage but also extent to the postmitotic neuron. We aim in expanding these preliminary results by examining for other parameters that may have been affected in the postnatal cortex by prenatal DEX exposure, such as the presence of cortical

[7] My personal contribution to this experiment was in designing the experimental setup, treating the animals and collecting and processing the brain tissue, whereas sectioning and pilot staining was performed by other colleagues.

interneurons and whether the morphology of the corpus callosum is altered due to the increased number of neurons, a change in gliogenesis or is a secondary effect from changes in fiber tracts.

Furthermore, pilot behavioral data from DEX-treated animals (adult group) suggest that DEX may induce long-lasting behavioral abnormalities in adulthood[8]. Adult animals (age 6-9 months) were trained in different tasks such as the elevated plus maze and the open field test to examine anxiety. Preliminary data indicate that the DEX exposed animals show decreased anxiety such as by spending more time in the center and the open arms of the elevated plus maze and in the center of the open field. Therefore, these results show that even a single course of DEX at a clinically relevant dose given during midgestation, premature initiates GR signaling and leads to permanent changes in the cortical cytoarchitecture and the behavior of the animal. We plan to expand these results by evaluating the animals for additional behavioral tasks that may reveal more differences that are more subtle and more specific for certain regions. For example, fear conditioning can be examined with the use of the step-down avoidance task and depression-related behavior can be examined with the tail suspension test.

5.1.2.2 *Gene regulation by the unliganded and DEX-activated GR in neural progenitors*

It would be interesting to see how GR regulates gene expression and homeostasis in RGCs and IPCs, in basal unliganded conditions and following DEX treatment on E14.5. To approach this we could use FACs analysis to specifically isolate RGCs and IPCs, in combination with RNA-seq analysis in samples of dorsal cortices that were derived from animals that were exposed to DEX or not (basal conditions). This would allow us to evaluate the transcriptional role of the unliganded GR in each of the progenitor population and compare how DEX affects it.

[8] My personal contribution to this experiment, was to perform the treatment of the animals, whereas the behavioral testing and data analysis was performed by other colleagues.

Furthermore this analysis can be conducted in yet another level of detail, by specifically dissecting the role of the unliganded GR in RGC and IPC progenitors that are in different phases of the cell cycle. These experiments are very important since they can reveal new gene targets of GR in neural progenitors that control their proliferation and differentiation. Furthermore, this analysis would allow us to build a more specific model on how GR differentially regulates the proliferation and differentiation of RGCs versus IPCs and reinforce our conclusions for a role of the unliganded GR in neurogenesis.

An additional analysis venue that we can follow is to evaluate the basal post-transcriptional modifications of GR in the RGCs versus IPCs, and specifically the phosphorylation status of GR in basal conditions and following DEX-treatment on E14.5. Phosphorylation of GR on Ser211 was shown to differ in a cell cycle-phase dependent manner in basal unliganded conditions. Furthermore, this Ser211 phosphorylation is maximal during late G1 phase and gives the unliganded receptor maximal responsiveness to GCs. Therefore, we could explore whether the abundance of Se211 GR phosphorylation differs in RGCs and IPCs that are in different cell cycle phases by FACs analysis, both in basal and after DEX exposure conditions, following the same setup as for the current study i.e., evaluate GR phosphorylation after 24 hours of DEX exposure on E14.5. Furthermore, we can use immunohistochemistry to examine whether Ser211 GR phosphorylation is detectable *in vivo* in RGCs and IPCs.

Another interesting question, is to see whether a single exposure to DEX on E15.5, which is the point that endogenous GC production starts in the mouse embryo, will lead to different effects on RGC and IPC proliferation by following a similar approach as we have done for this study and also by examining gene expression in RGCs and IPCs. Specifically it would be interesting to see whether the normally activated GR versus the now “ultra” activated GR (since

in addition to the endogenous GCs, DEX exposure will activate a greater complement of GRs), will lead in different effects on the competence of the progenitors. This analysis would allow us to determine whether GR has a different role in neural progenitors depending on the developmental stage and might explain how prenatal exposure to GCs may result in very different effects on brain development and lead to different clinical outcomes in neonates.

While our study was limited to examination of the effects of premature activation of GR signaling by DEX, we can expand our studies by examining *in vivo*, the effects of the other commonly used prenatal GC, betamethasone (BETA). While DEX and BETA, have closely related chemical structures, but they have different GR potency and pharmacokinetics (Tegethoff et al., 2009) which can lead to different clinical outcomes. Therefore, evaluating the effects of Beta on GR signaling properties and subsequently in brain development and neural progenitor proliferation and differentiation, would allow us to better understand how selecting a specific treatment can affect the developing brain and potentially create better GC alternatives that promote only the desired benefits in premature babies while eliminating the possible neurological side-effects.

APPENDIX A

DIFFERENTIAL SUBCELLULAR LOCALIZATION OF THE GLUCOCORTICOID RECEPTOR IN DISTINCT NEURAL STEM AND PROGENITOR POPULATIONS OF THE MOUSE TELENCEPHALON *IN VIVO*

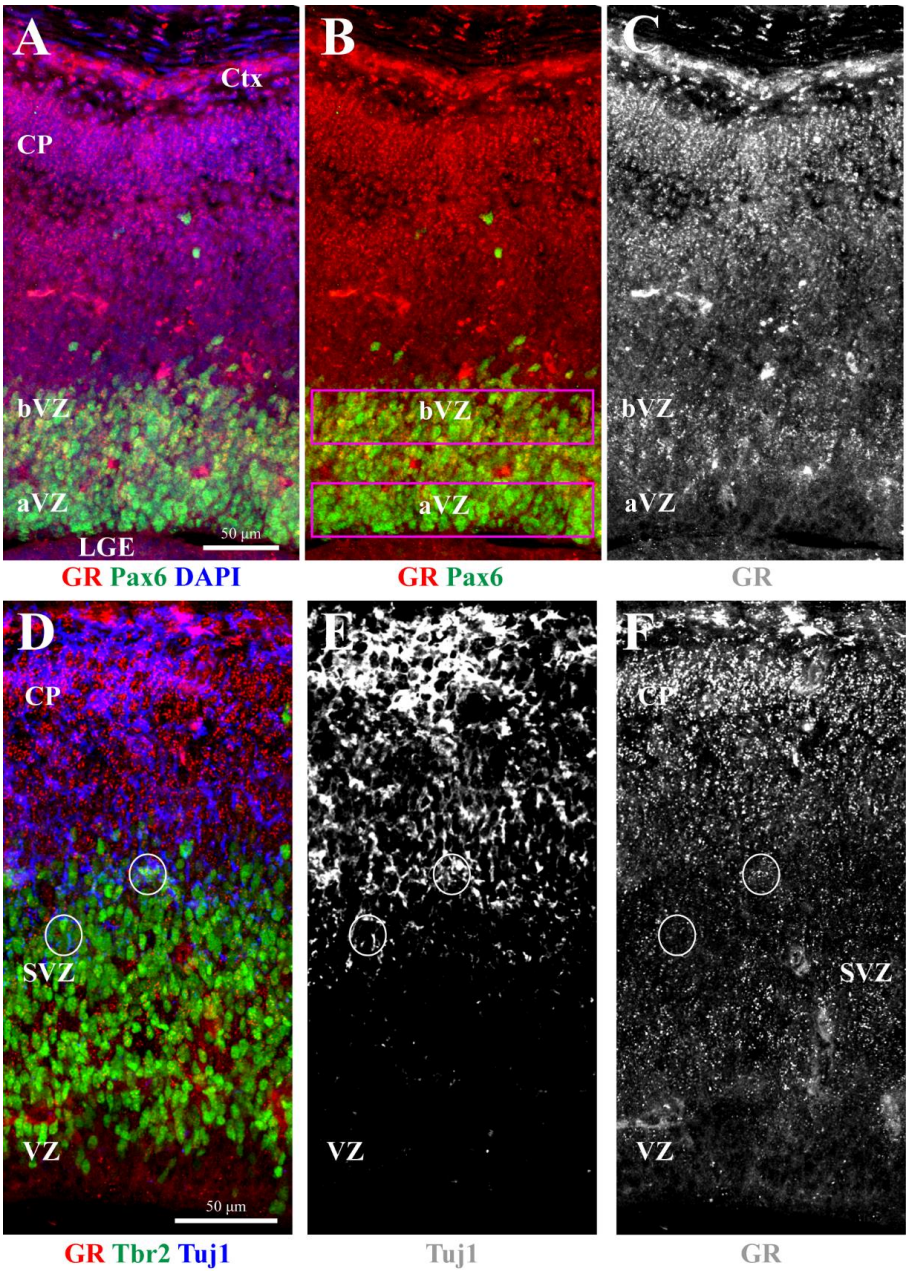


Figure 35. GR is expressed in NSPCs and neurons of the E11.5 dorsal telencephalon. (A) z-stack of a sagittal section of the dorsal telencephalon stained with BuGR2 (red) and Pax6 (green) and counterstained with DAPI (blue). (A, B) Pax6-positive RGCs in the ventricular zone express GR in an apical low, basal high gradient. Panel C shows only GR in grayscale. (D) z-stack of a coronal section of the dorsal telencephalon, containing part of the lateral ganglionic eminence (LGE) and stained for BuGR2 (red), Tbr2 (green) and Tuj1 (blue). PP, preplate; bVZ, basal VZ; Ctx, cortex.

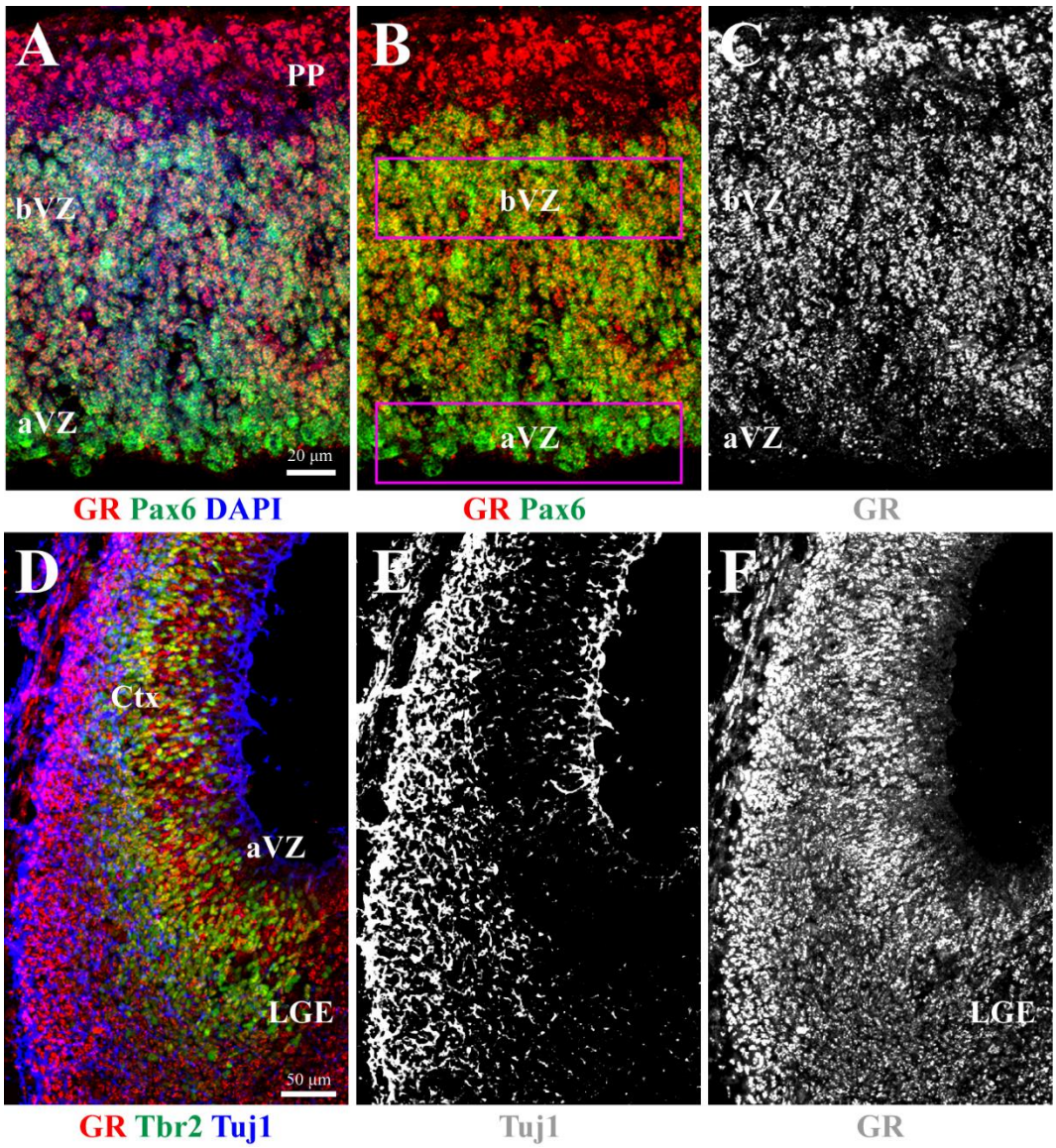


Figure 36. GR is expressed in NSPCs and neurons of the E13.5 dorsal telencephalon.

(A, D) z-stacks of sagittal sections of the dorsal telencephalon on E13.5, immunostained for BuGR2 (red) and Pax6 (green) (A) or Tbr2 (green) and Tuj1 (blue) (D). (A-C) RGCs on the apical surface express primarily cytoplasmic GR compared to RGCs in the basal ventricular zone (bVZ). The expression profile of GR (grayscale) is seen in panel C. (D-F) GR expression in IPCs and neurons (circles outline nascent neurons positive both for Tuj1 and Tbr2) is primarily nuclear. CP, cortical plate; Ctx, cortex; LGE, lateral ganglionic eminence; SVZ, subventricular zone.

APPENDIX B

PREMATURE ACTIVATION OF GR DURING MIDGESTATION ALTERS NEURAL PROGENITOR PROLIFERATION IN A CELL CYCLE DEPENDENT-MANNER AND IMPAIRS CORTICAL DEVELOPMENT

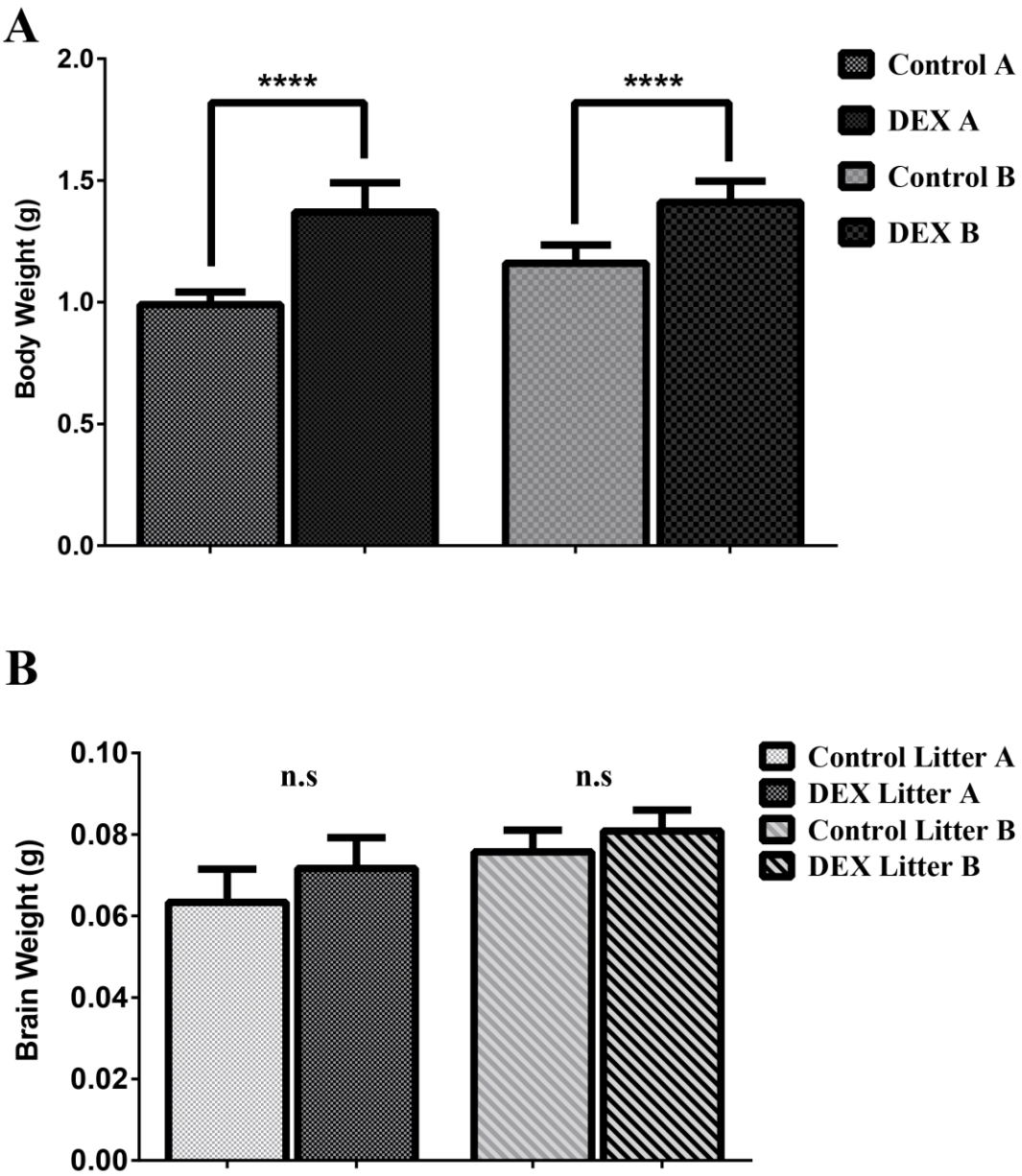

A
2.0
1.5
1.0
0.5
0.0
Body Weight (g)
****
****
Control A
DEX A
Control B
DEX B
B
0.10
0.08
0.06
0.04
0.02
0.00
Brain Weight (g)
n.s
n.s
Control Litter A
DEX Litter A
Control Litter B
DEX Litter B

Figure 37. A single exposure to DEX on E14.5, results in increased body weight on E17.5 but without an effect on brain weight. *A.* The body weight was increased at E17.5 in two independent litters following DEX treatment. Average ± SD; Litter A $n_{control}$=11, 0.99 ± 0.05; n_{DEX}= 6, 1.37 ± 0.12, $p < 0.001$; Litter B $n_{control}$=11, 1.16 ± 0.07; n_{DEX}= 12, 1.4 ± 0.09, $p < 0.001$. ***B.*** DEX treatment on E14.5 did not affect brain weight proportionally to the effects it had on the body weight.

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