HELLENIC REPUBLIC
National and Kapodistrian University of Athens
Department of Biology

Athens International Master's Programme in Neurosciences

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Center of Clinical Research, Experimental Surgery and Translational Research

RESEARCH THESIS PROJECT

THE ROLE OF CORTICOTROPIN-RELEASING HORMONE (CRH) IN HUMAN BRAIN DEVELOPMENT: AN ORGANOID STUDY

Theodorou Iliana

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Contents

The Role of Corticotropin-Releasing Hormone (CRH) in Human Brain Development: an Organoid Study

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Scientific Summary

Corticotropin-Releasing Hormone (CRH) was first identified as a neurohormone secreted by the hypothalamus in response to stressful stimuli. However, large amounts of CRH are also secreted by the placenta of anthropoid primates during pregnancy. Although the role of hypothalamic CRH has been extensively studied, there is a remarkable lack of evidence regarding the role of placental CRH. In order to investigate the effects of placental CRH on human brain development and to overcome the limitations raised in experimenting with human tissue, we have generated human 3D-neural spheroids and human cortical organoids from human embryonic stem cells (hESCs). Exposure of neural spheroids and cortical organoids to CRH results in significant differences in their size and cellular composition. In addition, immunohistological analysis using cortical layer-specific antibodies, revealed differences in the cytoarchitecture of the organoids exposed to CRH as compared to control. Pharmacological disruption of the CRH signaling, with the use of a CRH receptor antagonist named NBI, reversed the effects of CRH. In addition, RNA sequencing analysis of the CRH and NBI-exposed organoids revealed altered expression of genes related to neurodevelopmental processes. Our findings suggest that this *in vitro* approach provides a unique tool for our understanding of the mechanisms underlying the role of placental hormones in human brain physiology.

Highlights

- ✓ Exposure of neural spheroids and cortical organoids to CRH led to significant differences in their size and cellular composition
- ✓ Immunohistological analyses using cortical layer-specific antibodies revealed differences in the cytoarchitecture of CRH-exposed organoids compared to the control group.
- ✓ Pharmacological disruption of the CRH signaling, using the specific CRH receptor 1 antagonist NBI, leads to faster cellular differentiation
- ✓ RNA sequencing analysis of CRH- and NBI-treated organoids unveiled altered expression of genes related to neurodevelopmental processes
- ✓ RNA sequencing analysis of CRH- and NBI-treated organoids revealed altered expression of genes involved in Retinoic acid metabolism

Keywords

CRH, Placental CRH, Human Brain Development, 3D-neural spheroids, Cortical Organoids

Lay Summary

Maternal environment plays a crucial role in pregnancy, exerting significant influence on the complex processes that shape fetal brain development. From the early stages of pregnancy, maternal factors, ranging from nutritional cues to hormonal variations, and stress responses, impact the trajectory of fetal brain development. This symbiotic relationship highlights the vital importance of comprehending how both endogenous and exogenous maternal environment sculpts the neural landscape, establishing the foundation for the offspring's cognitive, emotional, and physiological well-being throughout life. Among these exogenous factors, maternal hormones, particularly Corticotropin-Releasing Hormone (CRH), secreted by the placenta, critically affect fetal brain development. The unique secretion pattern of CRH from the placenta of only humans and anthropoid primates poses challenges for the use of traditional mammalian models, leading to the utilization of human brain organoids as an

innovative alternative. While brain organoids provide a miniature representation of physiological human brain development, certain differences, such as the absence of vascularization and interactions with other cells, exist. Investigating the impact of CRH on human brain development through exposure of human brain organoids to large concentrations of CRH or to a drug that blocks the action of CRH, revealed that both over-exposure and absence of CRH result in abnormal development. These findings suggest a pivotal role of CRH in shaping human brain.

Introduction

The human brain stands as one of the most intricate systems in the organism, with the cerebral cortex comprising over 80% of its total mass (Azevedo *et al.*, 2009). The cerebral cortex is presumed to have pivotal role in brain physiology by controlling all conscious and unconscious functions such as perception, thought, attention, voluntary movement, intelligence, and personality. These cognitive abilities and sensorimotor skills require a well orchestrated and strictly regulated neuronal network, characterized by appropriate migration and positioning of neurons, acquisition of layer-specific transcriptional hallmarks, and formation of precise axonal projections, procedures occurring at the early embryonic stages. In vertebrates neural fate is induced in the ectoderm. Through the process of neurulation a pool of neuro-epithelial(NE) cells emerge in the neural tube (Tam and Loebel, 2007). These NE cells serve as precursors to all cells in the central nervous system (CNS), including the cerebral cortex. Early in neurogenesis, NE cells undergo morphological, mitotic, and molecular changes, leading to the generation of a new population of progenitor cells known as radial glia (RG). Radial glia is a distinct cell type characterized by apical-basal processes and plays a crucial role in the correct formation of the six-layered cortical architecture, serving as a "ladder" for the migration of newly generated neurons. The radial migration of newborn neurons ultimately gives rise to the radial organization of the mature cortex in a birth-date-dependent inside-out manner (Noctor et al. 2001, Arai and Taverna 2017). This inside-out model of neurogenesis describes the sequential generation and migration of neuronal populations in the developing cortex, with deeper layers forming first and being progressively overlaid by later-generated superficial layers. This process contributes to the establishment of the layered structure of the mature cerebral cortex.

In the early stages of development, radial glia (RG) cells undergo symmetric divisions during a phase known as "neural expansion," effectively expanding the pool of daughter cells with a

progenitor cell fate (Noctor et al. 2004, Haubensak et al. 2004). During the peak of neurogenesis RG cells progressively begin to divide asymmetrically to produce progenitor cells with early neuronal cell fate, while maintaining symmetrical divisions (Malatesta, Hartfuss, and Götz 2000 ,Tamamaki et al. 2001). Depending on the location of these mitotic divisions, RG cells can be further classified as apical radial glia (aRG), located in the ventricular zone (VZ) (Gal *et al.*, 2006) or basal radial glia (bRG) located in the subventricular zone (SVZ) (Tabata, Yoshinaga and Nakajima, 2012) (Figure 1). The identification of cortical progenitor cells transitioning from apical and basal progenitors to post-mitotic neurons can be achieved by studying the expression of molecular markers, including Pax6 (apical progenitors), Tbr2 (basal progenitors), and Tbr1 (neurons) (Englund et al. 2005, Kawaguchi et al. 2008).

Recently, a distinct subtype of radial glia (RG) cells has been identified, characterized by a modified radial morphology and localization to the subventricular zone (SVZ). These cells are known as outer radial glia (oRG) and are notably prevalent in gyrencephalic mammals (Hansen *et al.*, 2010), Fietz et al. 2010, Reillo et al. 2011). The oRG cells show radial morphology and express classic markers of radial glial progenitor cells including Pax6(Götz, Stoykova and Gruss, 1998), phospho-vimentin (Bignami, Raju and Dahl, 1982), GFAP (glial fibrillary acidic protein), and BLBP (brain lipid-binding protein) (Feng, Hatten and Heintz, 1994), which distinguishes them from typical basal progenitors.

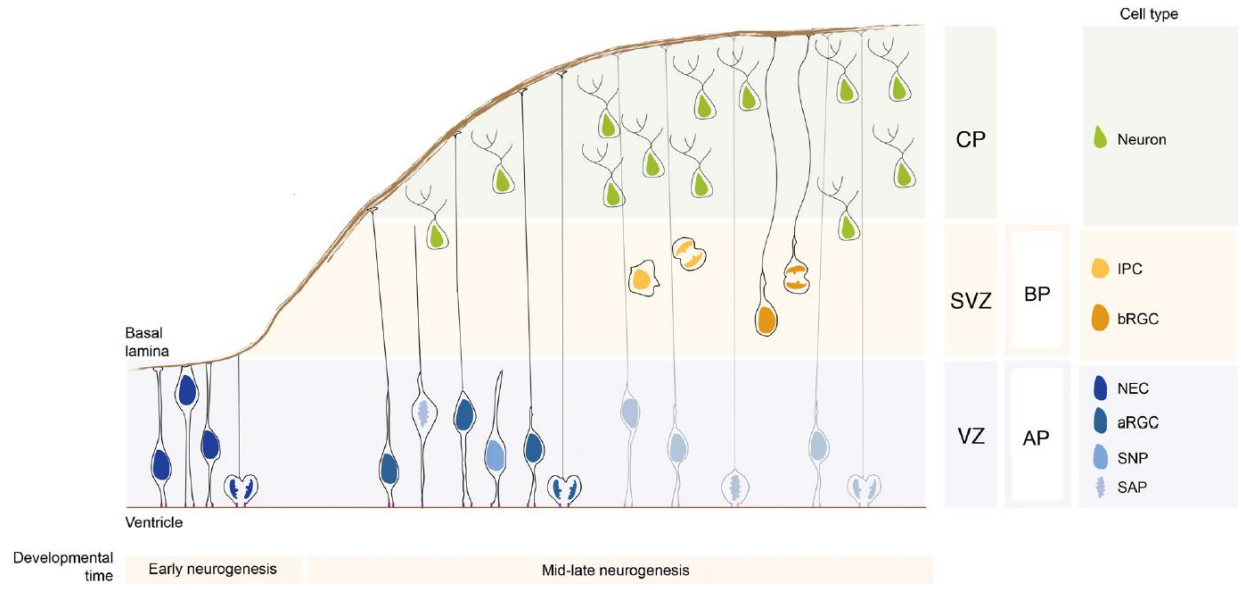


Figure 1. Neural stem and progenitor cell types in the developing neocortex. During early neurogenesis, neuroepithelial cells (NECs) forming the ventricular zone (VZ) are responsible for the lateral expansion of the neocortex. During mid-late neurogenesis, apical progenitors (APs) divide and give rise to basal progenitors (BPs), which form a new proliferative zone, the

subventricular zone (SVZ). The APs pool is mainly composed of apical radial glial cells (aRGCs) and a lower proportion of short neural precursors (SNPs) and sub-apical progenitors (SAPs). The BPs pool is composed by intermediate progenitor cells (IPCs) and basal radial glial cells (bRGCs). APs and BPs give rise to neurons that migrate basally and settle in the forming cortical plate (CP).(borrowed by Arai and Taverna, 2017)

Finally, at the late stages where neurogenesis reaches completion, a "gliogenic switch" prompts neural stem cells to generate astrocytes and oligodendrocytes in the neocortex (Clavreul et al. 2019, Gao et al. 2014). These types of glia constitute more than half of the total cells of the CNS (Herculano-Houzel, 2014), and play indispensable roles in neural development and functioning. Astrocytes, a type of glial cell, serve various functional roles, including maintaining the blood-brain barrier (BBB), providing physical and trophic support to neurons, guiding axons, participating in synapse formation and remodeling, modulating synaptic transmission, regulating osmotic pressure, and maintaining ion homeostasis (Khakh and Deneen, 2019). On the other hand, the primary function of oligodendrocytes is to form myelin sheaths around axons, facilitating the rapid propagation of action potentials and providing trophic support for the myelinated axons (Nave and Werner, 2021). Similar to neurons and progenitor cells, specific molecular markers have been identified to better understand the structure, function, and roles of these cells in the CNS. Notably, OLIG2 is a frequently used marker for cells of the oligodendrocyte lineage, while GFAP serves as a commonly used marker for astrocytes (Huang *et al.*, 2023) (Table 1).

Stem-like or precursor cells	**Astrocytes**	**Neurons**	**Oligodendrocytes**
SOX2 (VZ, neural stem cells)	Glial fibrillary acidic protein (GFAP)	Neuronal tubulin (beta 3 tubulin)	O4(immature)
Nestin (neural stem cells, radial glia)		Microtubule-associated protein 2 (MAP2) (expressed in dendrites)	O1(more mature)
GLAST (VZ, neural stem cells, radial glia)			Myelin basic protein (MBP)
BLBP (Brain lipid binding protein) (radial glia)			Olig1/2(precursors and mature)

(Borrowed by Hall and Miller, 2011)

Table 1. Neural Cell Markers

It is evident that the stringent regulation of every stage in cerebral cortical development is necessary for the successful progression of this highly complex and organized process. Any disruption during these diverse stages may lead to malformations in cortical development, with the severity of the abnormalities dependent on the specific developmental stage affected. While certain disorders like microcephaly, megalencephaly, or cortical dysplasia stem from

anomalies during development, there are syndromes with a multifactorial pathogenesis. The role of the mother in this developmental process is particularly crucial, as the fetal brain is influenced by the local microenvironment, beginning with fundamental nutrients as building blocks and extending to hormonal regulation. The developing embryo is exposed to maternal factors through the placenta or bloodstream, and any abnormalities in this environment can result in impaired cortical development. Consequently, this can give rise to various neurodevelopmental disorders, including but not limited to schizophrenia, autism, epilepsy, and others (Estes and McAllister 2016 ; Schmitt et al. 2014 ; Van Den Bergh, Dahnke, and Mennes 2018).

Among the hormones secreted by the human placenta, Corticotropin-releasing hormone (CRH) holds a significant role. CRH is a 41-amino acid neuropeptide primarily and mainly synthesized in the paraventricular nucleus (PVN) of the hypothalamus. Its main function is to regulate the hypothalamus-pituitary-adrenal gland axis (HPA) and orchestrate the physiological response to stress (Vale *et al.*, 1981). During stress response, CRH is released from the hypothalamus, triggering the secretion of vasopressin (AVP) from the posterior pituitary gland. Subsequently, CRH binds to receptors in the anterior pituitary gland, inducing the production of adrenocorticotrophic hormone (ACTH). ACTH is then released into the bloodstream, stimulating the adrenal cortex to produce glucocorticoids—cortisol in humans and corticosterone in mice. (Behan et al. 1995; Fowden et al., n.d.).

CRH is also produced by the placenta and fetal membranes during pregnancy, playing a pivotal role in determining the length of gestation, the timing of parturition, and influencing developmental trajectories (McLean *et al.*, 1995). Notably, from the end of the 1st trimester to the end of gestation, placental CRH production undergoes a 40-fold increase, reaching levels comparable to those expected in the hypothalamic portal system during stress (Davis and Narayan, 2020). Despite the structural and functional similarities between placental and hypothalamic CRH, there are distinctions in the regulation of their expression. Specifically, hypothalamic CRH is negatively regulated by cortisol, while placental CRH production is elevated in response to elevated cortisol levels. In prior research from our group, it was demonstrated that CRH plays a crucial role in mouse brain development, with CRH deficiency leading to neurogenic abnormalities during embryogenesis (Koutmani *et al.*, 2013). Additionally, recent reports have indicated a direct local action of CRH in both embryonic and adult hippocampal neural stem cells (NSCs), involving multiple well-characterized signaling pathways (Koutmani *et al.*, 2019).

In humans, the deregulation of the CRH/CRH receptors' system has been linked to the development of psychiatric disorders. Additionally, epigenetic changes in the CRH gene have been correlated with a general psychiatric risk score in adolescents (Jokinen *et al.*, 2018). Furthermore, exposure to abnormal concentrations of placental CRH during both early and late gestation has been associated with distinct patterns of cortical thinning (Sandman, 2018) and has been implicated in cognitive and emotional deficits later in childhood (Sandman *et al.*, 2018).

Considering the aforementioned factors, a more comprehensive understanding and investigation of the role of the hormonal factors in brain development is essential for the development of new diagnostics and clinical applications. Until recently, knowledge about human brain formation relied on non-invasive indirect techniques, such as ultrasound imaging, and animal models. Rodents have been a common model for understanding the mechanistic pathways implicated in malformations of cortical development through genetic manipulation, as they share many similarities with humans. However, rodents are lissencephalic animals thus lacking the abundance of different BPs and the extensive expansion of the SVZ and of the CP (Dehay, Kennedy, and Kosik 2015, Betizeau et al. 2013). To address these limitations, other gyrencephalic species such as ferrets and sheep are used. However, due to challenges in maintenance, cost, and differing genetic backgrounds, the development of alternative models is imperative.

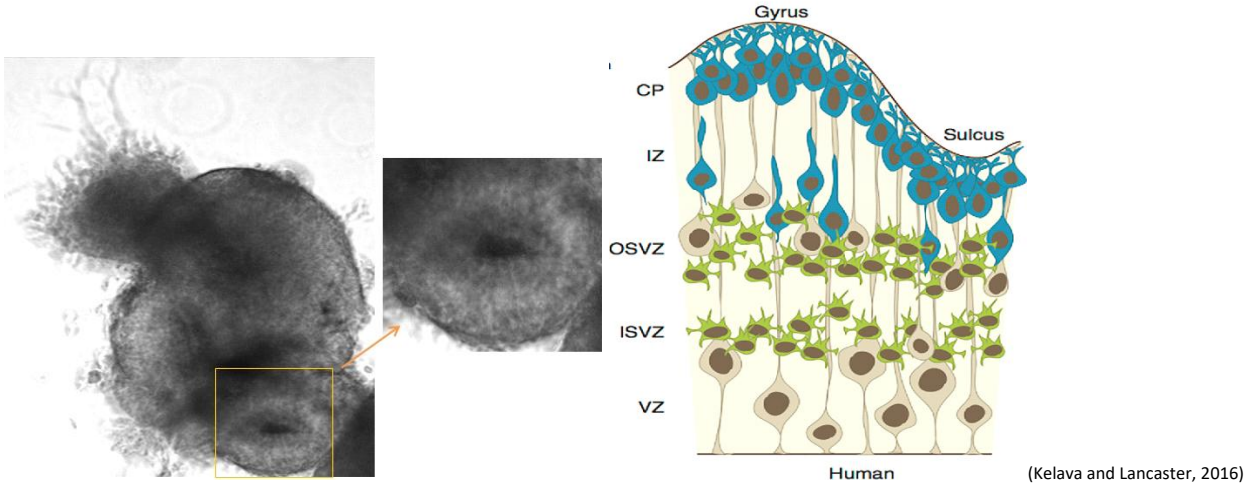
To address this, recent technological advances have enabled the creation of a novel in vitro three-dimensional (3D) model known as organoids. These organoids are generated from cultured embryonic stem cells or induced pluripotent stem cells (iPSCs) (reviewed in: Chen *et al.*, 2020), Eichmüller and Knoblich 2022, Agboola et al. 2021). This innovative approach allows for the simulation of human brain formation and provides insights into the development of various brain regions.

Brain organoids, or cerebral organoids, cortical organoids, hippocampal organoids etc., are not models of the whole human brain, but of specific brain regions. While they may not capture all aspects of the developing cortical cytoarchitecture, they do produce all cell types present in the cerebral cortex in a layer-specific manner, resembling the mature human cortex. Additionally, being derived from human stem cells, they carry the genetic landscape of humans.

Different ways exist to generate these human 3D models; one of them refers to homogeneously looking aggregates called “neurospheres”. In this model, stem cells are left to self-assemble first into embryoid bodies (EBs) and later into “neural spheres” called

"neurospheres". Subjecting neurospheres to external developmental factors results in the generation of cells that differentiate into all neuronal subtypes but also into astrocytes and oligodendrocytes, thereby mimicking the normal cell fate of neural progenitors. The proliferative and differentiating capabilities of neurospheres make them an excellent model for studying neurogenesis and neural development, albeit in a non-region-specific manner.

Another system of 3D brain tissue, closely emulating the endogenous developmental program, is referred to as cerebral organoid. This model relies on the inherent ability of stem cells to self-organize into intricate tissue structures. The effectiveness of this system in mimicking human neocortical development is based on the exogenous application of specific growth factors and signaling pathway inhibitors at precise time points (Kadoshima et al. 2013, Lancaster and Knoblich 2014). At first, stem cells are left to self-assemble into embryoid bodies (EBs) and then they are embedded in Matrigel that supports the formation of a polarized neuroepithelium. *In vivo*, the polarized neuroepithelium of the neural plate will fold to form a rounded neural tube, a structure that has not yet been replicated in organoids. Thus, the use of Matrigel mimics the generation of these structures by giving rise to neural tube–like "buds" in which neuroepithelial cells organize radially around large apical lumens (Benito-Kwiecinski and Lancaster, 2020) (Figure2).



(Kelava and Lancaster, 2016)

Figure 2. A ventricle formed in a 20- days organoid embedded in Matrigel

In contrast to neurospheres, cortical organoids exhibit a fundamental layering of the cortical plate, with deep layer neurons positioned closer to the apical side and upper layer neurons closer to the basal side. Cerebral or cortical organoids are believed to replicate the timing of corticogenesis during the first trimester of human gestation, offering a reliable system for

modeling the development of the human cortex (a 18–40 days developmental period in cerebral organoids models a 21–42 days post-conceptional period in humans) (Kadoshima *et al.*, 2013).

However, organoids also exhibit certain limitations. Firstly, there is a degree of variability between the sizes of the ventricles that are developed, while all layers are compressed into a confined space. Additionally, overlaying tissue and meninges are absent, vascularization and body axes are not formed, and, in most protocols, glial cells are scarce. Despite these limitations, 3D human models representing different regions of the developing brain are valuable due to their capacity to generate all cell types found in gyrified species within a 3D architecture. Organoids serve as a valuable tool, complementing animal models, to explore the development of the cortex in health and disease and in response to various stimuli.

In our study, we investigated the impact of CRH on human brain development, utilizing 3D-neural spheroids and cerebral cortical organoids derived from human embryonic stem cells (hESCs). Our findings reveal that exposure of neural spheroids to CRH, along with pharmacological disruption of CRH signaling, induces changes in the proliferation and fate of neural stem cells. We also present evidence demonstrating that CRH affects gene expression and architectural features in human cerebral cortical organoids at different developmental stages. More specifically, immunohistological analysis reveals that overexposure or absence of CRH force neural stem cells to exit earlier from the cell cycle and undergo differentiation at an accelerated rate compared to the control group. Moreover, RNA sequencing analysis depicts a disrupted metabolic pathway of Retinoic Acid, both under CRH- and CRH receptor's antagonist-exposure conditions, further supporting our observations. These results highlight the pivotal role of CRH in human brain development and propose that this *in vitro* approach serves as a distinctive tool for comprehending the mechanisms that underlie the involvement of hormones in human brain physiology. Additionally, it paves the way to intriguing possibilities for pharmacological applications in the context of neurodevelopmental disorders.

Methods

Human Embryonic Stem cells

Human embryonic stem cell (hES) line HUES6 (with a normal 46-XX karyotype, kindly provided by Dr R. Matsas, Hellenic Pasteur Institute) and H9 cell line (with a normal 46-XX karyotype) were used. The derivation and characterization of the H9 cell line were previously described (Thomson et al., 1998).

Culture

Human Embryonic Stem Cells

Both human ES cell lines were cultured on mitomycin- mitotically inactivated mouse embryonic fibroblasts. H9 cells were cultured in culture medium consisting of 80% KnockOut Dulbecco's modified Eagle's medium, an optimized medium for mouse ES cells (Gibco BRL, Rockville, MD), 1 mM L-glutamine, and 1% nonessential amino acids (Gibco BRL), supplemented with 20% KnockOut SR, a serum replacer optimized for mouse ES cells (Gibco BRL). The components of KnockOut SR have been published elsewhere (Price et al., 1998). For the culture and maintenance of HUES6 cell line, mTESR1 Basal Medium supplemented with 1x mTESR1 Supplement (StemCell Technologies) was used.

Generation of Human Neural Spheroids

Human neural spheroids were generated as previously shown by Kobolak *et al* (Kobolak *et al.*, 2020) and Chambers *et al* (Chambers *et al.*, 2009) with some modifications. Briefly, human neural progenitor cells (hNPCs) derived from either H9 cell line or HUES6 cell line, were generated using the synergistic action of two SMAD inhibitors, LDN-193189 (Abcam) and SB431542 (StemCell Technologies). Additionally, floor plate induction was further followed by the treatment of two agonists of the hedgehog signaling pathway, Purmorphamine (Stemgent) and SAG (Abcam) (at days 6-16) according to the protocol of Kriks *et al* (Kriks *et al.*, 2011)

Generation of Cortical Organoids

Human cortical organoids were created as previously shown by Lancaster *et al* (Lancaster and Knoblich, 2014) by HUES6 cells.

More specifically, after the confluency of feeders reached the desired levels, hES cells were treated with collagenase IV (StemCell Technologies) for 10 minutes at 37^{o} C, followed by scraping with a cell lifter to remove intact colonies and trituration to obtain smaller colonies before plating. For inactivation of Collagenase IV, equal volume of hES medium was added and the total amount of medium was centrifuged at 800 rpm for 2 minutes. Cell pellet was resuspended in 1 ml of hES medium containing low-bFGF (final concentration 4 ng/ml) and ROCK inhibitor (1:100, final concentration 50 μM). Additional appropriate volume of low-bFGF hESC medium with ROCK inhibitor was added in order to obtain 9,000 live cells per 150 μl. 150 μl were plated in each well of a low-attachment 96-well plate and left for incubation until they form embryoid bodies (EBs). When EBs reached 350–600μm in diameter, ROCK inhibitor and bFGF were excluded from the medium.

After the EBs reached ~500–600 μm in diameter and began to brighten and have smooth edges, neural induction was performed by neural induction medium [DMEM-F12(Life Technologies) with 1% N2 supplement (ThermoFischer Scientific), 1% GlutaMAX supplement (Invitrogen), 1% MEM-NEAA (Sigma) and heparin (final concentration of 1 μg/ml)] for 6 days. Approximately on day 14 (depending on EBs morphology), EBs were embedded in Matrigel drops and cultured in cerebral organoid differentiation medium (DMEM-F12, Neurobasal medium, N2 supplement, insulin, GlutaMAX supplement, MEM-NEAA, B27 supplement and penicillin-streptomycin) for 19 days. At this step, Matrigel drops were separated in 3 different groups, the control group, the CRH group (containing CRH at a concentration of 10^{-7}M) and the NBI group (a CRH-R antagonist, at a concentration of 10^{-6}M). Finally, after a total of 47 days in culture the cortical organoids were analyzed by immunostaining and RT-qPCR.

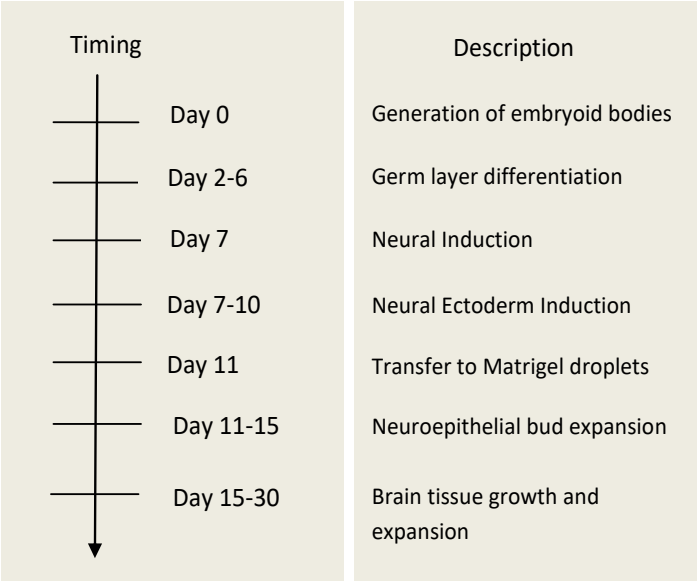
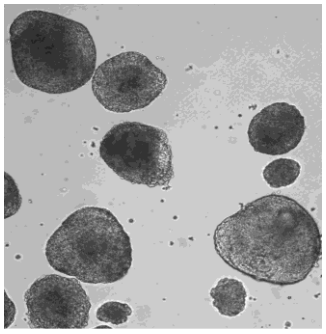
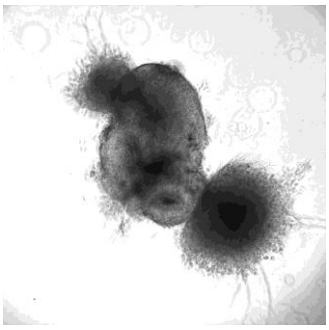


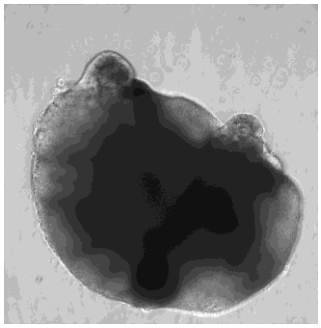
Figure 3. Timing and description of cortical organoid formation



Embryoid bodies day 2



Cortical organoid day 15



Cortical organoid day 24

Human neural spheroids culture

For the generation of neural spheroids, HUES6 cell line was used. More specifically, undifferentiated hES cells were cultured in one well of a six-well plate coated with feeders [(CF-1 MEF IRR 2M, (Gibco)] in human embryonic stem cell medium(DMEM/ F12, 20% Knockout™

Serum Replacement (KO-SR), 1% penicillin /streptomycin and 1% Non-essential amino acids) supplemented with the ROCK inhibitor Y-2763210. After 72 h, cells were transferred to Neuronal Induction Medium containing DMEM/ F12, 1% N2, 2% B27, 1% penicillin /streptomycin, LDN-193189 and SB431542 and allowed to differentiate for a total of 7 days. On day 7, cells were exposed to a combination of SAG and Purmorphamine for induction of the Floor-Plate. After approximately 10 days neurospheres were formed and after reaching a similar size, they were separated in 3 groups (Control, CRH, NBI) and left for expansion in neural maintenance medium (NMM; DMEM-F12, supplemented with 1% N2and 2% B27 and 1% penicillin /streptomycin) until the desired maturation stage. Neurospheres were left for 45 days in culture and then stored at -80 for cryosectioning.

Cryosectioning

For the organoids that were embedded in Matrigel, fixation with 4% PFA was performed in the wells. First, the medium was aspirated and then 4% PFA was added for 1 hour at RT. PFA was discarded and the organoids were washed with PBS for 10 minutes twice. Organoids from each condition were transferred in falcons containing 30% (wt/vol) sucrose solution for cryopreservation and placed at 4 °C overnight to allow tissues to sink into sucrose solution. The next day, after organoids have sunk into sucrose, they were transferred in OCT and stored at -80°C. For immunofluorescence, 40 µm cryosections were taken on SuperFrostTM slides.

The same procedure was conducted for cryoprotection of neurospheres.

Immunostaining

The sections were post-fixed with 4% PFA for 10 min at RT. Sections were permeabilized with 0.3% Triton in PBS and blocking was performed in 10% normal goat serum (NGS) blocking solution for 1h at RT. Blocking solution was used to dilute the primary and secondary antibodies whereas nuclei were visualized using 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Primary antibodies were incubated for 16-24 h at 4^{o}C and subsequently sections were exposed to secondary antibodies for 1 h at RT. Fluoroshield™ with DAPI (Sigma-Aldrich) was used for mounting of the sections.

For BrdU (Sigma-Aldrich, Cat# 858811) antigens, an additional DNA hydrolysis step was required to allow access of the anti-BrdU (Abcam) antibody within the DNA. First, cells are labeled *in vitro* with BrdU (10 µM) for 2 hours in RT and washed with PBS. DNA hydrolysis followed by addition of 2N HCL for 15 minutes in 37^{o}C which was then neutralized with 0.1 M sodium borate buffer pH 8.5 for 20 minutes at RT. Finally, immunostaining was conducted according to

standard immunocytochemistry protocol as described above.

TUNEL assay was performed using the DeadEnd™ Fluorometric TUNEL System (Promega). First, cells were incubated *in vitro* with TdT reaction mixture for 60 minutes at 37°C and then reactions were terminated by adding 2X SSC buffer in the culture for 15 minutes. Cells were then centrifuged at 300 × g for 10 minutes at 4°C and the medium was removed, taking care to avoid aspirating the cells. Cells were washed in fresh PBS for 5 minutes at room temperature and analysis of the immunostaining was conducted according to standard immunocytochemistry protocol as described above.

Microscopy and Image analysis

Fluorescent stainings were visualized using a Leica laser-scanning microscope and analyzed with FIJI ImageJ. For analysis of the hCOs, only ventricles that fulfilled the following criteria were analyzed: clear ventricular structures with elongated, radially-organized cells surrounding the ventricular zone (VZ- determined either with DAPI, SOX2 or PAX6 staining).

REAGENTS	COMPANY	CAT. No
CRH	Tocris	1151
BD Matrigel Matric Basement Membrane	BD Biosciences	354234
Adenosihe-cyclic Monophosphate (cAMP)	Millipore Corporation	28745
NBI	Sigma Aldrich	N3911
DMEM High Glucose 4,5g/L	Invitrogen	31966-021
FBS	Gibco	10270-064
Penstrep-400	Sigma Aldrich	P4333
Glutamine	Gibco	25030-024
Knock-out SR Serum Replacement	Gibco	10828-028
Non-essential Amino Acids	Invitrogen	11140-035
DMEM/F12 without glutamine	Gibco	21331-020
Mitomycin C	Sigma	M4287
Collagenase IV	Gibco	17104-019
PBS	Gibco	14190-094
Recombinant Human FGF basic (bFGF)	Bio-technce	233-FB-025/CF
Rock Inhibitor	Calbiochem	6880001MG
B-27 Supplement (without vitamin A)(50X)	Gibco	17504-044
N2 Supplement (100X)	Invitrogen	107502-048
LDN193189	STEMCELL technologies	72142
SB-4331542	Cayman Chemical	13031
Retinoic Acid (RA)	Sigma Aldrich	R2625-50MG
Smoothened Agonist (SAG)	Millipore Corporation	566660
Purmorphamine	STEMCELL technologies	72204
CHIR99021	STEMCELL technologies	72052
Neurobasal	Gibco	21103-049
Recombinant Human GDNF	PeproTech	450-02
Recombinant Human BDNF	PeproTech	AF-450-10

Table2. Reagents and recombinant proteins used in cell culture

NAME	MANUFACTURER, CAT No	DILUTION
GFAP	Cell Signaling, 3670	1:300
Olig-2	Millipore Corporation, AB9610	1:400
Nestin	Santa Cruz, sc-23927	1:300
Sox2	Abcam, ab97959	1:400
B III Tubulin	Covalence, MMS-435P-250	1:500
vGlut1	Millipore Corporation, MAB5502	1:300
CRH	Abcam, ab272391	1:300
CRHR1	Santa Cruz, sc-5543	1:300

Table3. primary Antibodies

NAME	MANUFACTURER,	DILUTION
Mouse 488	Alexa Fluor™	1:500
Mouse 533	Alexa Fluor™	1:500
Rabbit 488	Alexa Fluor™	1:500
Rabbit 568	Alexa Fluor™	1:500

Table 4. Secondary Antibodies

RNA isolation

Total RNA was isolated from day 30 hCOs, in duplicates with 3-4 organoids per replicate, either treated with CRH or NBI for 18 days. RNA extraction was performed using RNeasy Mini Kit (50). For reverse transcription, 500 ng of the isolated RNA was used applying the M-MLV Reverse Transcriptase (Thermo Fischer scientific) according to the manufacturer's instructions. The quantification of RNA was performed with Nanodrop (IMPLEN).

Bulk RNA sequencing

Library preparation

RNA-Seq experiments were conducted at the Greek Genome Center (GGC) of the Biomedical Research Foundation of the Academy of Athens (BRFAA). RNA-Seq libraries were prepared with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, with 1μg total RNA input. Library QC was performed with the Agilent bioanalyzer DNA1000 kit and quantitation with the Qubit HS spectro photometric method. Sequencing was performed in NovaSeq 6000. Approximately 25 million 100 bp Single-End reads were generated for each sample.

Analysis

Quality Control (QC) was performed at the FASTQ raw data file for each sample using the FASTQC software. FASTQ files were aligned to mm10 mouse genome using HISAT2. Counts were defined using HTSeq htseq-count command with the "intersection non-empty" option (Kim, Langmead and Salzberg, 2015). The count files were used as input for DESeq2 (Anders, Pyl and Huber, 2015) Normalization was performed with the estimate size factor function followed by Differentially Expressed Genes Analysis (DEGs) analysis. Pathway analysis was performed at EnrichR environment (Chen *et al.*, 2013; Kuleshov *et al.*, 2016; Xie *et al.*, 2021). Heatmaps were constructed with pheatmap package in R, after computing the respective z-score. Dotplots were constructed with ggplot2 package in R. Volcano plots were constructed using the Volcano Plot design tool in Galaxy.

Results

CRH signaling disruption affects proliferation and neurogenesis in human neural spheroids

To assess the impact of placental CRH on human brain development, we first employed simple 3D models, namely neural spheroids. Human 3D-neural spheroids were generated in accordance to the protocol outlined by Kobolak et al. (2020) following neural induction of the human embryonic stem cell line H9, according to the protocol of Chambers modified by Fasano and Kriks (Chambers et al., 2009; Fasano et al., 2010; Kriks et al., 2011) (Figure 1).

After 30 days in culture, the stem cells within the spheroids undergo differentiation, yielding neurons, while the appearance of astrocytes and oligodendrocytes is observed not earlier than 50 days of culture.

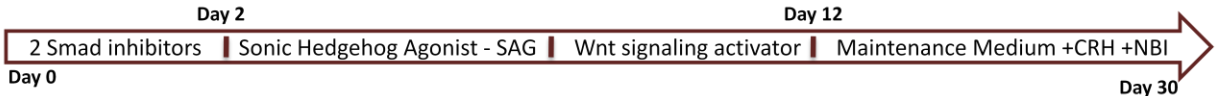


Figure 1|Schematic representation of the experimental design of the different stages of human 3D-neural spheroids' generation

Preliminary experiments conducted in our laboratory have revealed that CRH is not only endogenously secreted by neural spheroids but also plays a crucial role in their development. More specifically, the inhibition of CRH signaling pathway has been found by our group to reduce neurogenesis, particularly affecting the production of glutamatergic neurons while promoting astrogliogenesis (data not shown). These experiments were conducted on mature spheroids at 50 days, prompting us to explore cellular distribution at an earlier developmental stage, at day 30 of spheroid maturation.

Thus, after 30 days of culture, we observed that disruption of the CRH signaling pathway results in a reduction of the proliferating activity of neural stem cells, as evidenced by BrdU incorporation assay. Nestin-positive cells were notably reduced only following complete blockade of the CRH pathway (Figure 2A, 2B) while the number of differentiated neurons (β-tubIII+ cells), (Figure 2C) remained unaffected by CRH signaling alterations. In order to investigate the effects of CRH on the apoptotic activity of neural stem cells, we performed TUNEL assay and we observed no effect of CRH at this developmental stage (Figure 2D)

Collectively, these findings portray an altered developmental profile in which the proliferation and neurogenic potential of neural stem cells within CRH- and NBI-treated human neural

spheroids are reduced, suggesting a faster transition towards a gliogenic phenotype based on our previous observations of induced gliogenesis at 50 days of culture.

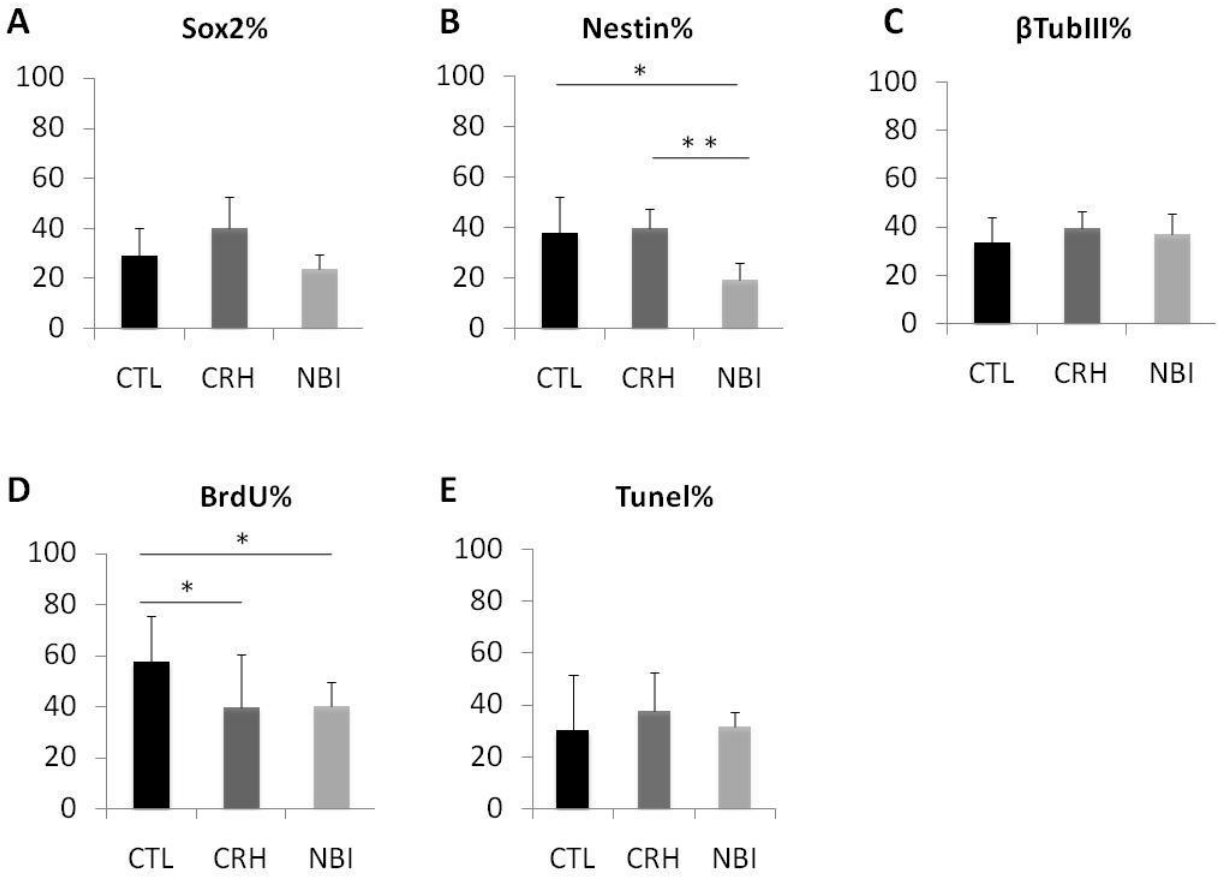


Figure 2 | Neurogenesis and gliogenesis are altered in CRH and NBI-treated human neural spheroids **A.** Quantification and comparison of the percentage of Sox2 positive cells between the 3 groups. **B.** Quantification and comparison of the percentage of Nestin positive cells between the 3 groups. **C.** Quantification and comparison of the percentage of β-tubIII positive cells between the 3 groups. **D.** Quantification and comparison of the percentage of BrdU positive cells between the 3 groups. **E.** Quantification and comparison of the percentage of Tunnel positive cells between the 3 groups. Data represent the mean ±s.e.m (n=8). P values for **A** to **E** as indicated by asterisks, were calculated using 2-way ANOVA with Benjamini testing correction. *P<0.05, **P<0.01.

CRH signaling alterations affect early corticogenesis

Neural spheroids provided insights into the critical role of CRH signaling in determining cell fate of neural stem cells within the neural lineage. In order to delve deeper into the biological

implications of placental CRH in more complicated developmental processes, such as the establishment of cortical layers, and to examine its effects on cytoarchitecture, we proceeded to the generation of cortical organoids. Utilizing the human embryonic stem cell line HUES6, we followed established protocols to achieve the formation of these organoids (Figure 3).

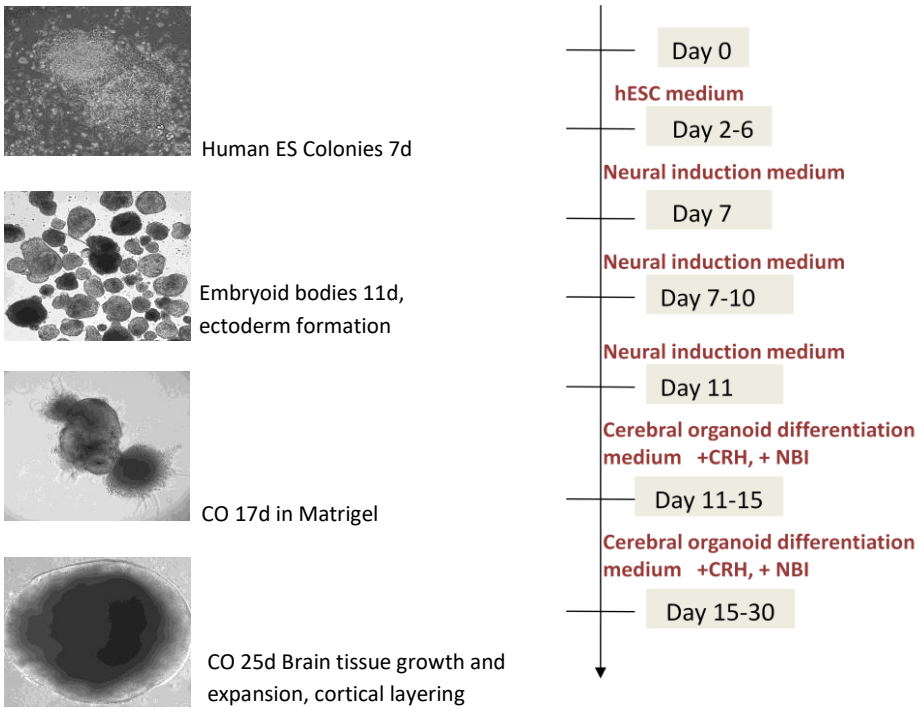


Figure 3| Schematic representation of the experimental design.

After 30 and 45 days in culture, cortical organoids exhibited the formation of ventricular structures at their periphery (Figure 4A, 4B). At both developmental stages, distinct layers became evident: a zone surrounding the lateral ventricle (LV) exhibiting characteristics to the ventricular zone (VZ) and an outer zone resembling the cortical plate (CP) (Figure 4C). The VZ consists of undifferentiated neural stem cells and the CP consists of either mature neurons or neural progenitor cells that have not yet been differentiated. Finally, development of the subventricular zone (SVZ) is also discrete. The SVZ is characterized by the expression of Tbr2 a marker of Intermediate Progenitors (IPs) (Fig. 4D). At day 45 of cerebral organoids development, all the progenitor cell types are present, deep layer neurons' neurogenesis is at its peak and upper layer neurons' neurogenesis has started. Consequently, this time point

provides an opportune window for monitoring the effects of CRH and NBI on multiple cell types within the developing cytoarchitecture.

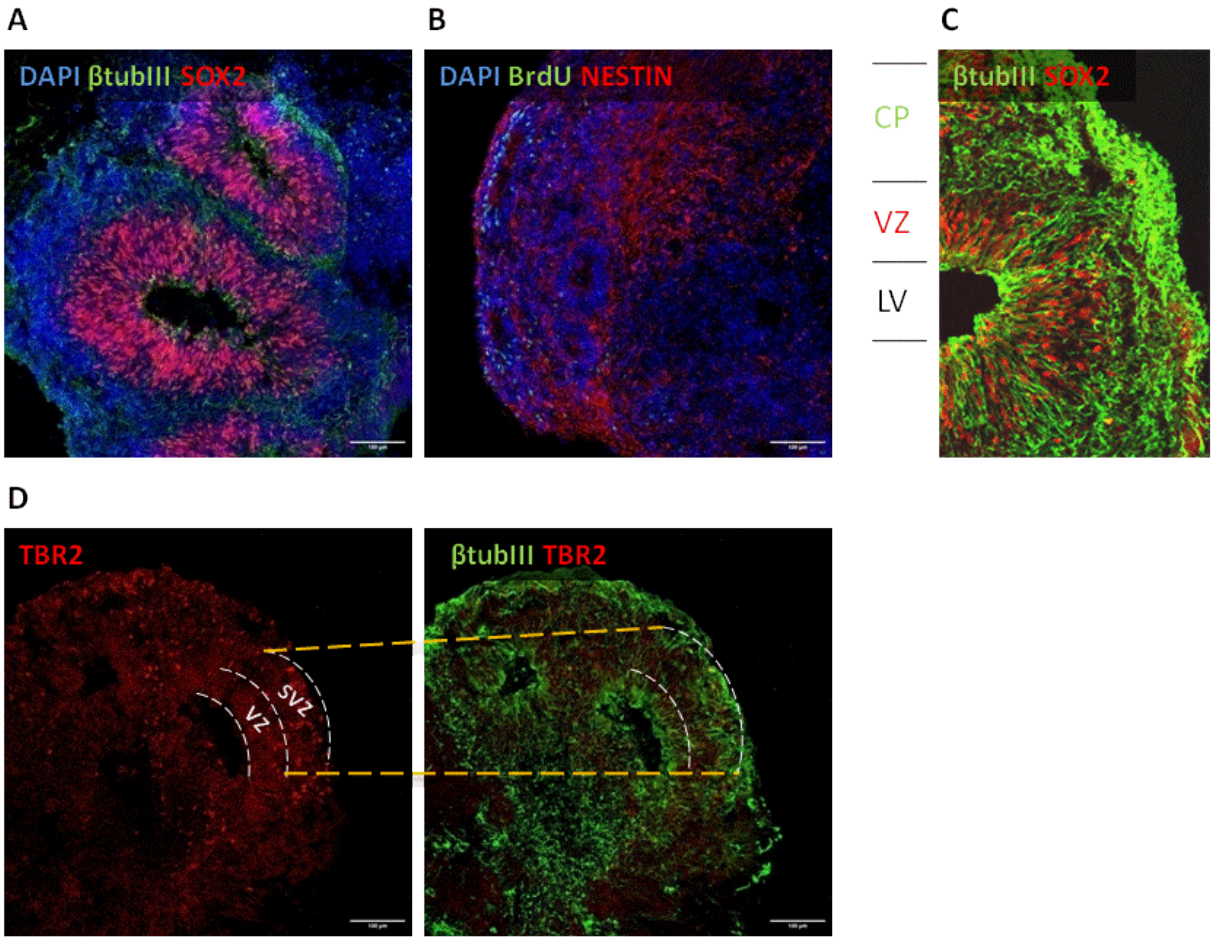


Figure 4 | Markers for developmental stages in Cortical Organoids. A. Representative image of 30-days human cortical organoid with characteristic ventricular structures and detection of SOX2+ and β-tubIII+ cells by immunofluorescence using anti-SOX2 and anti-β-tubIII antibody respectively. DAPI was used as counterstain. **B.** Representative image of 45-days human cortical organoid with characteristic ventricular structures and detection of Nestin+ cells by immunofluorescence using anti-Nestin antibody. DAPI was used as counterstain. **C.** Representation of the distinct layers (CP: cortical plate, VZ: ventricular zone, LV: lateral ventricle) formed inside the organoids and detection of the undifferentiated stem cells and mature neurons by immunofluorescence, using anti-SOX2 and anti-β-tubulin antibodies respectively. **D.** Representation of the Subventricular Zone (SVZ) formed inside the organoids and detection of the Intermediate Progenitor cells by immunofluorescence, using anti-Tbr2 antibody. Scale bars=100 μm.

We first assessed the impact of CRH at the early stages of corticogenesis, specifically at 30 days of organoid development. Interestingly, our findings revealed a modified pattern of neurogenesis at this developmental stage. Measurements of proliferating SOX2+ neural progenitor cells indicated an enlarged ventricular zone (VZ) in organoids that were exposed to CRH or NBI, as compared to the control group (Figure 5A, 5B).

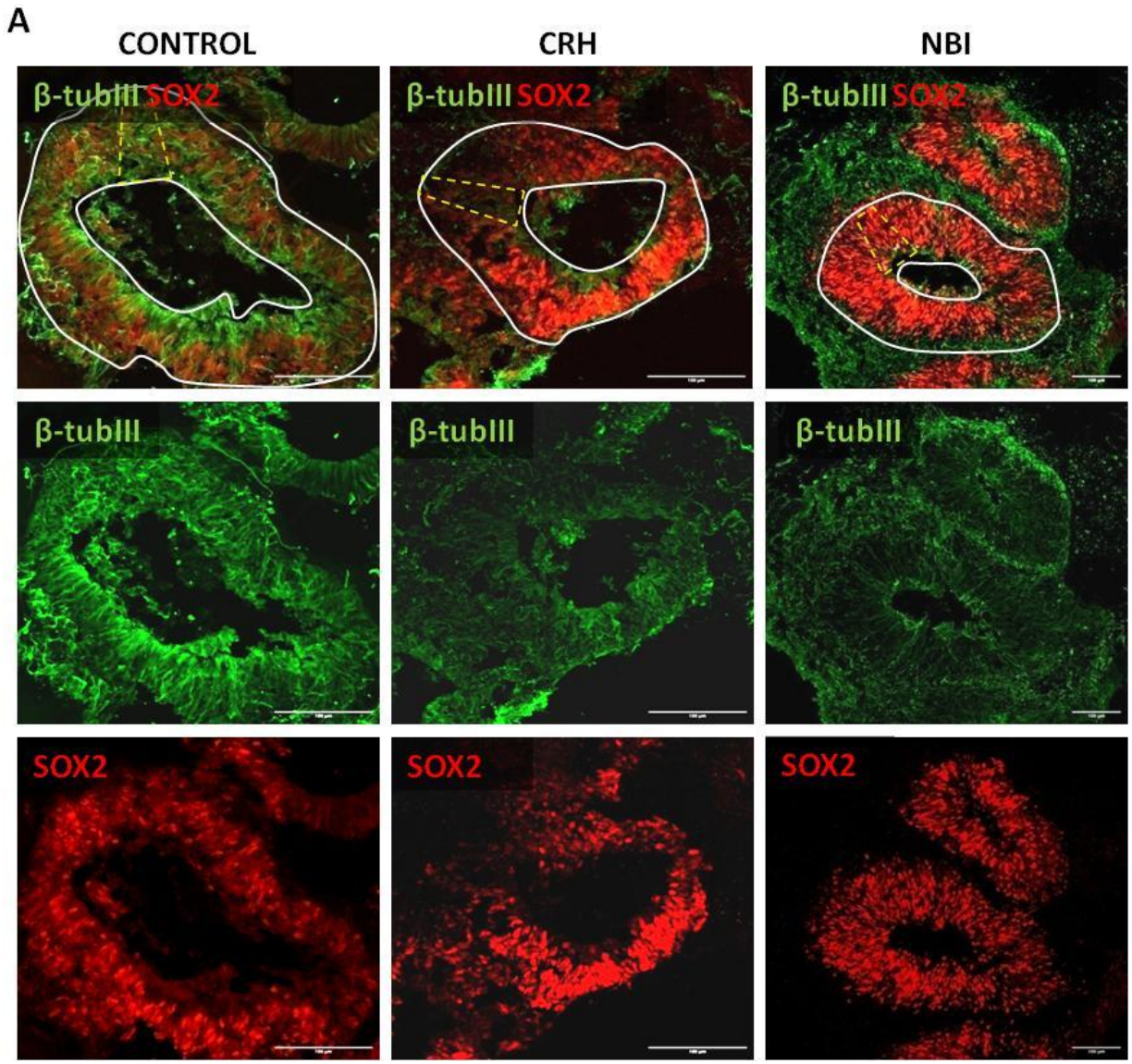


Figure 5 | CRH signaling is implicated in corticogenesis A. Representative images of day 30 hCOs at control, CRH and NBI conditions stained for SOX2 and β-tubIII. Dotted lines depict the measurements of the VZ in each condition. Scale bars, 100μm.

Nevertheless, the overall size of the ventricles in NBI-treated organoids surpassed that of the other experimental conditions (Figure 6A-C) suggesting an altered neural stem cell behavior regarding their exit of the cell cycle and the initiation of neuronal differentiation.

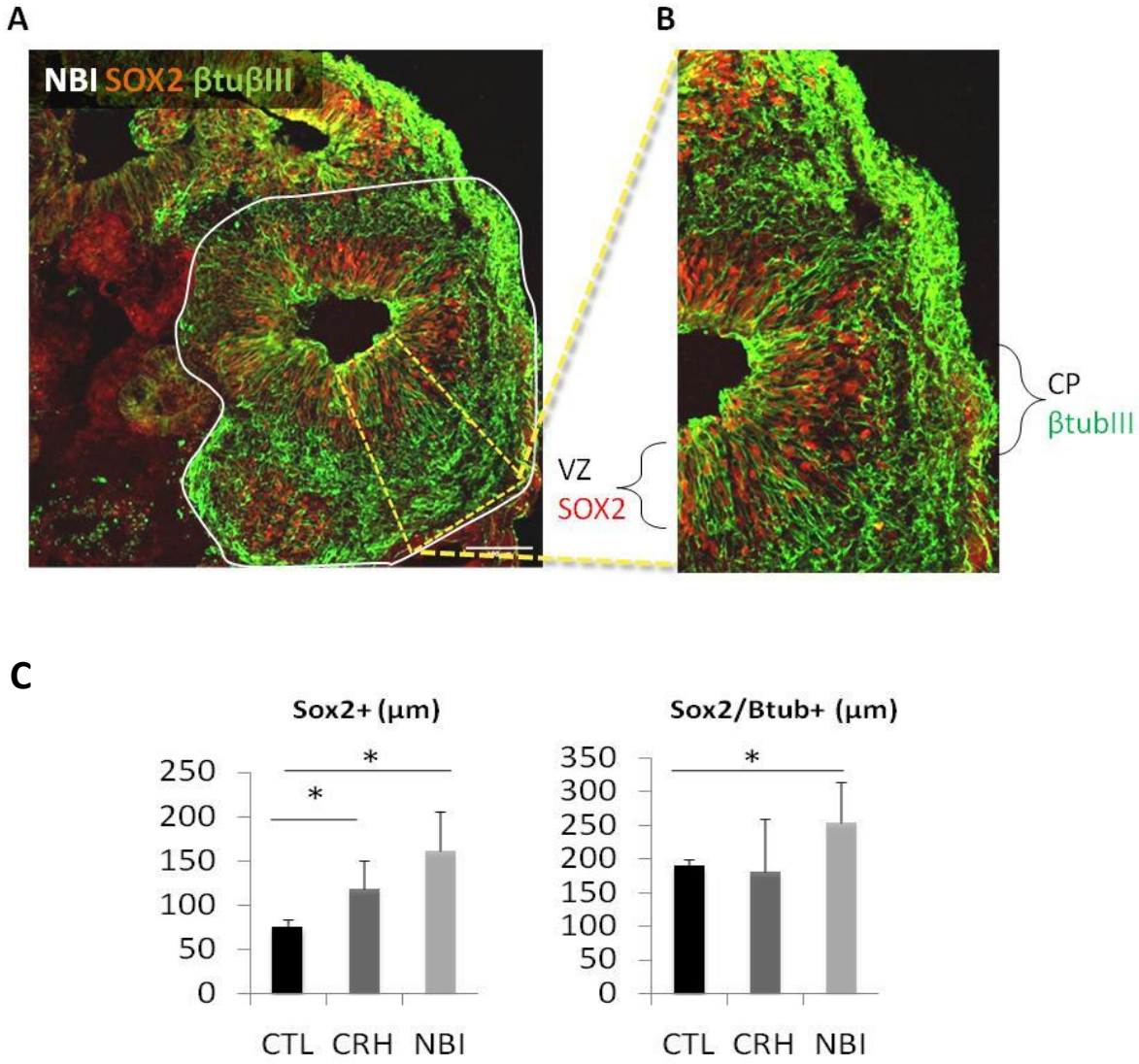
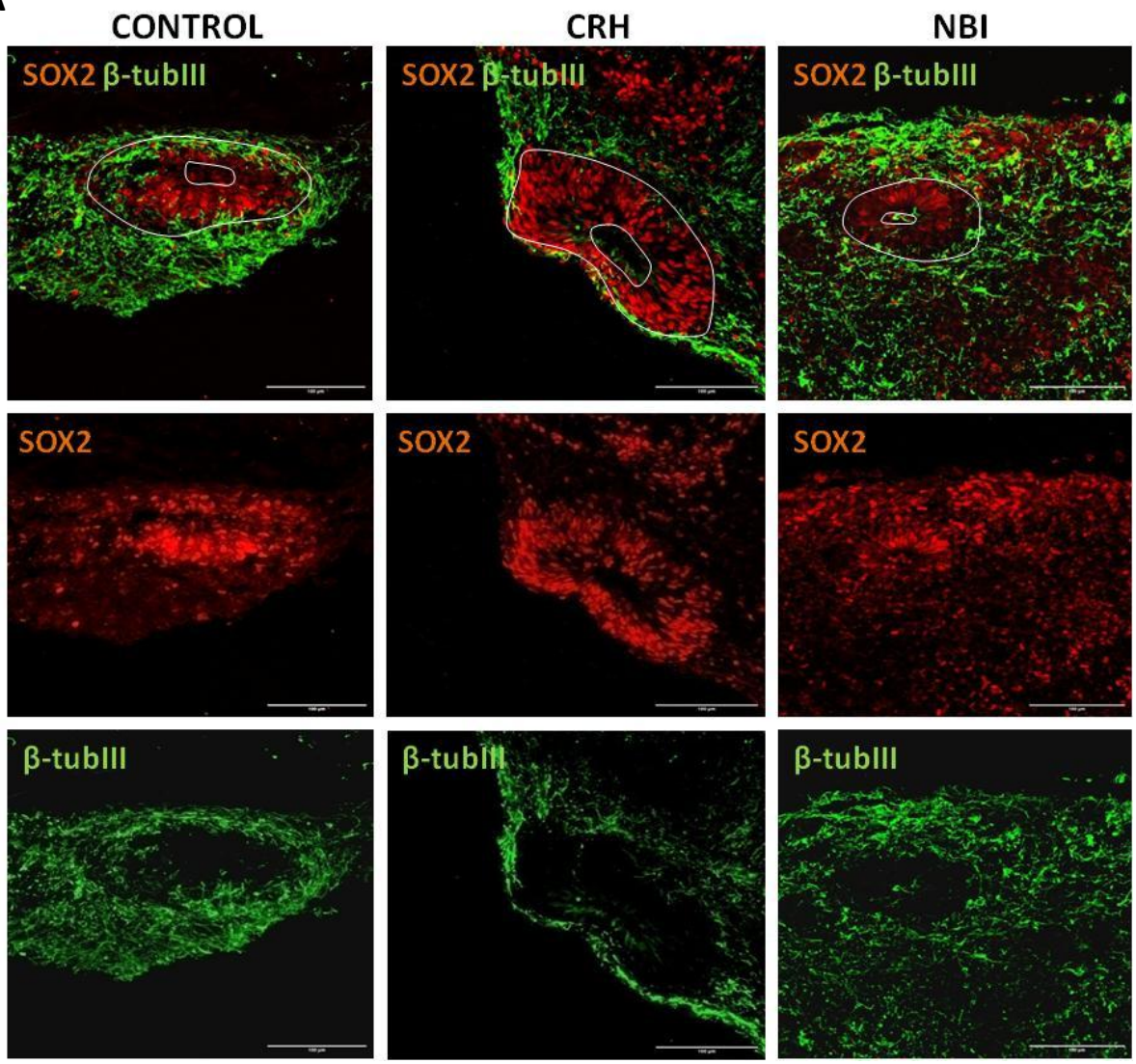


Figure 6| CRH signaling is implicated in corticogenesis A. Representative image of day 30 hCOs stained for SOX2 and β-tubIII. Dotted lines depict measurement from the inner of VZ to outer of CP. Scale bars, 100μm **B.** Zoom-in of the areas shown in dotted lines **C**. Quantification of the ventricle size in each treatment condition normalized by μm of quantified total area in hCOs. Data represent the mean ±s.e.m. (n=4). P values as indicated by asterisks, were calculated using 2-way ANOVA with Benjamini testing correction. *P<0.05.

CRH affects neuronal maturation at later developmental stages

As maturation progresses, on day 45 of development, the observed phenotype of CRH and NBI-treated cortical organoids undergoes reversal as compared to the 30 days organoids. More specifically, we observed a significant reduction of the size of the ventricular zone (VZ) and a decrease in progenitor cells under CRH- and NBI-treated conditions compared to the control group (Figures 7A, 7B) suggesting that any disruption in the CRH signaling pathway results in premature cellular differentiation. These findings substantiate and reinforce our earlier data obtained from the neural spheroids, emphasizing the crucial involvement of CRH signaling in corticogenesis.

A



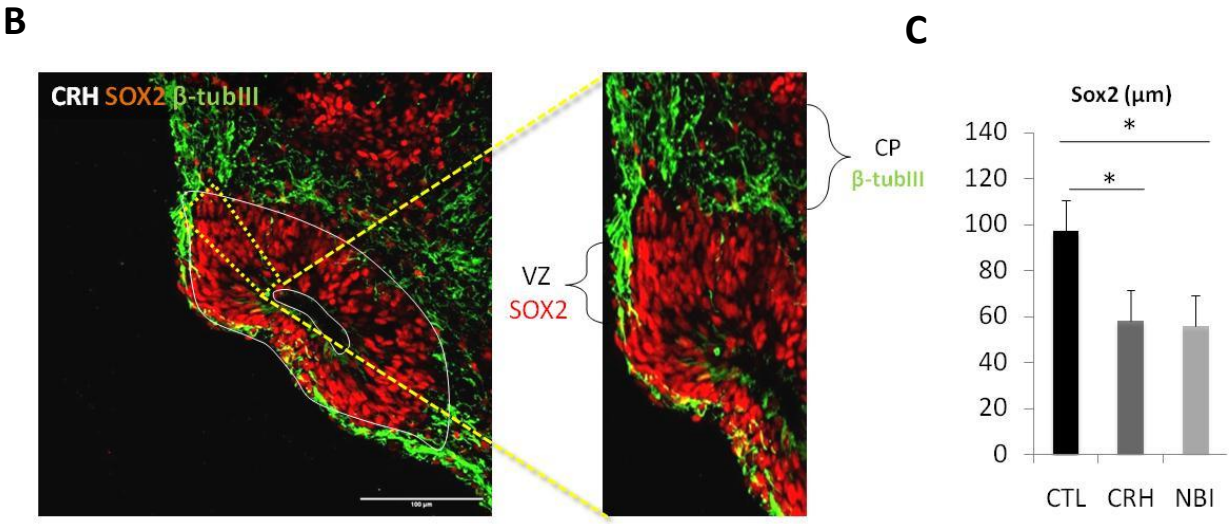


Figure 7 |CRH signaling alters corticogenesis at later developmental stages A. Representative images of day 45 hCOs at control, CRH and NBI conditions stained for SOX2 and β-tubIII. Dotted lines depict the measurements of the VZ in each condition. Scale bars, 100μm **B.** Zoom-in of the areas shown in dotted lines **C.** Quantification of the progenitor cells in each treatment condition normalized by μm of quantified total area in hCOs. Data represent the mean ±s.e.m. (n=4). P values as indicated by asterisks, were calculated using 2-way ANOVA with Benjamini testing correction. *P<0.05.

CRH Signaling affects transcriptional dynamics in human cortical organoids cellular landscapes

Having observed the phenotypic outcomes of manipulating CRH signaling, we sought to pinpoint the corresponding molecular-level correlations. To elucidate the genes and pathways potentially responsible for the accelerated cell exit, we conducted bulk RNA sequencing on both control and CRH or NBI-treated organoids. The analysis of differentially expressed genes(DEGs) revealed 13 DEGs in the Control vs CRH comparison and 66 DEGs in the Control vs NBI comparison, with 10 genes shared between these conditions (Figure 8A). Interestingly, most of the DEGs are related to Retinoic Acid metabolism and patterning according to literature (Figure 8B, 8C). Next, we proceeded to pre-ranked gene set enrichment analysis (GSEA) using the Gene Ontology Biological processes (GO-BP) database and we found a significant enrichment of gene sets associated with regulation of Retinoic acid signaling pathway in both CRH- and NBI- treated conditions, further supporting our DEGs (Fig 9A, 9B). These are interesting findings since Retinoic acid is a key signaling molecule during cortical development, suggesting a Retinoic acid-mediated role of CRH in human brain development.

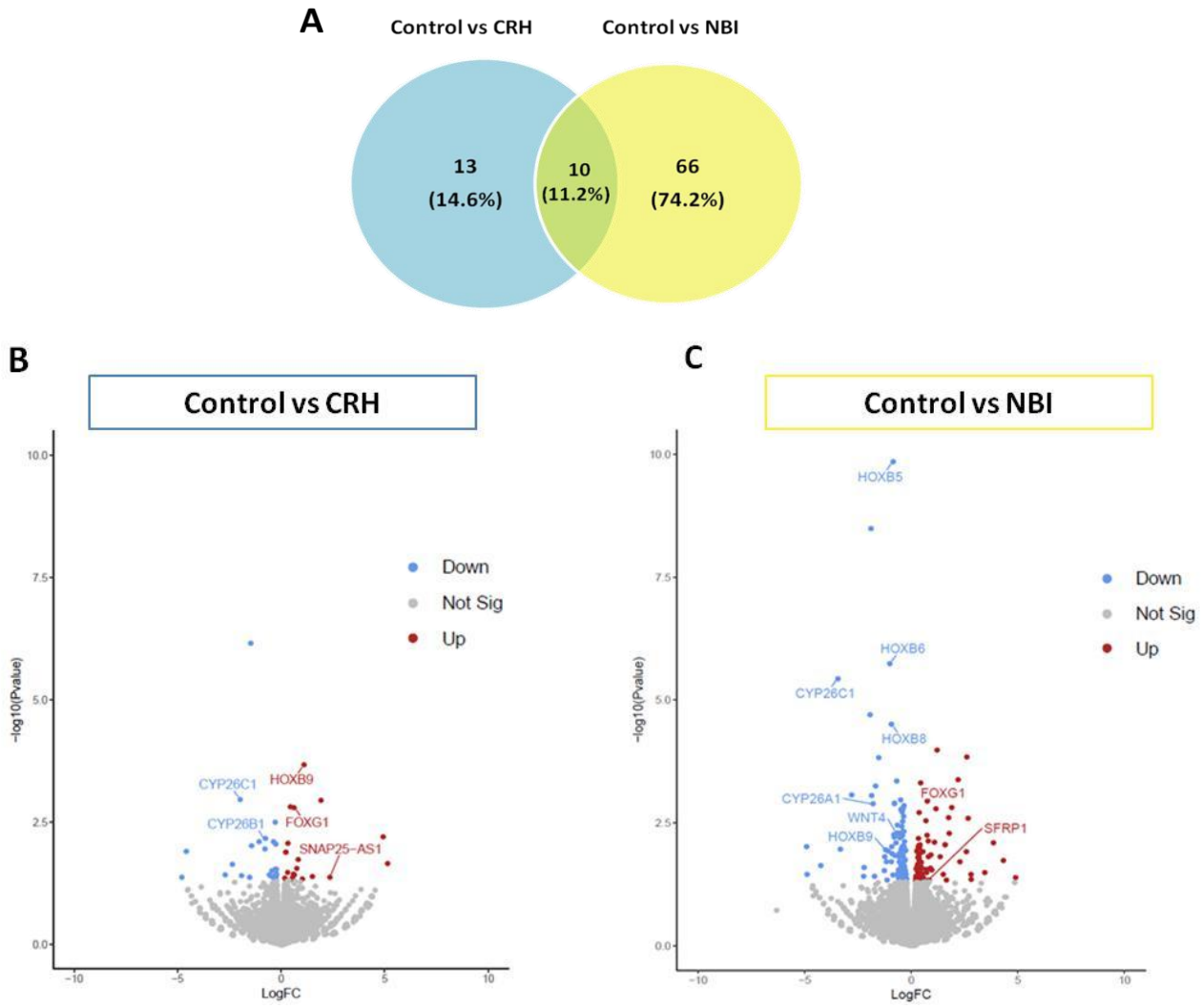


Figure 8| RNA sequencing analysis A. Venn diagram of DE genes from bulk RNA seq in 30-days control vs CRH-treated hCOs and control vs NBI- treated hCOs **B.** Volcano plot of the results of the bulk RNA seq. control vs CRH **C.** Volcano plot of the results of the bulk RNA seq. control vs NBI. Grey dots, genes with non-significant expression changes at an FDR cutoff of 10%; Blue dots, genes with downregulated expression at an FDR cutoff of 10%; Red dots, genes with upregulated expression.

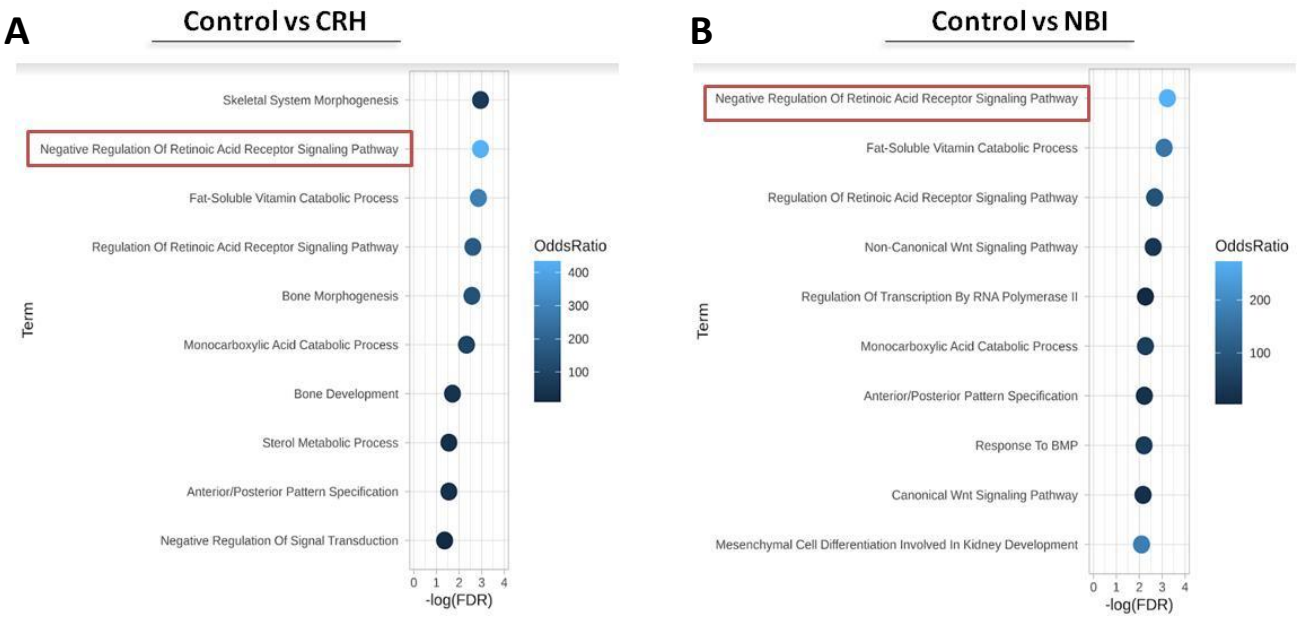


Figure 9 | Gene ontology analysis A. Top 10 biological processes enrichment analysis for the Control vs CRH group **B.** Top 10 biological processes enrichment analysis for the Control vs NBI group; FDR, false discovery rate.

Discussion

Undoubtedly, human brain development stands as one of the most complex processes, characterized by the remarkable ability to generate over 100 billion neurons from the initial 3-millimeter neural tube. This extraordinary feature highlights the absolute necessity for a strict regulation throughout the entire developmental process. Disruption at any stage of this process, whether influenced by internal or external factors, can have detrimental effects on the fetal brain, extending into childhood and beyond. Consequently, studying and comprehending the molecular mechanisms behind this complex system is crucial, as it contributes to enhanced prevention and detection of disorders affecting fetal brain development. Despite efforts to address this knowledge gap through the use of various animal experimental models, their inadequacy persists due to substantial differences with humans. Luckily, a recently developed and very promising model has been generated for the study of human brain development- the brain organoids. In our study, we generated cortical organoids to overcome the limitations associated with the exclusive secretion of placental CRH in humans and study its correlation with fetal brain development. In this study we identified CRH as an important factor in corticogenesis that exerts direct effects on human neural stem cells, altering their proliferative

properties and their commitment to the neuronal lineage. These findings suggest the possibility for potential therapeutic use of CRH in humans, in line with CRHR1 antagonists already undergoing clinical trials for CNS diseases (Dunlop *et al.*, 2017).

Since its discovery by Wylie Vale and colleagues in 1981 as a neurohormone pivotal for stress response, CRH has not only been found to be secreted in the hypothalamus but also identified in the placenta during human pregnancy (Sasaki, Shinkawa and Yoshinaga, 1989). Extensive research has primarily characterized placental CRH as a marker influencing gestational length and as a key initiator of human parturition. Clinical studies further associate disruption in the CRH/CRH receptors' system with the development of psychiatric disorders, distinct patterns of cortical thinning, and cognitive and emotional deficits in childhood (Kassotaki et al., 2021; Makrigiannakis et al., 2018a; Makrigiannakis et al., 2018b; Sandman, 2018; Sandman et al., 2018). Despite this clinical focus, molecular-level investigations remain scarce with some studies reporting a potential neuroprotective role for CRH by promoting neurogenesis, differentiation and survival of neuronal cells (Koutmani *et al.*, 2019; Koutmani *et al.*, 2013). In our research study, we demonstrated that manipulating CRH expression, either through overexpression or by total inhibition of its action, had a profound impact on the neurogenic period of progenitor cells in human neurospheres. This manipulation resulted in reduced proliferating ability, faster cell cycle exit, and accelerated differentiation. Interestingly, our findings differ from previously published data, which indicated a neuroprotective role for CRH in neural stem cells isolated from mouse brains. Unlike those studies, we did not observe an increase in apoptosis, suggesting potential differences in the resistance abilities of neural stem cells deriving from different organisms. Our results suggest that CRH may play a dual role in human neural stem cell physiology: it decreases the number of progenitors that have high proliferative and neurogenic potential and plausibly they confine the duration of neurogenesis period.

The data derived from human neural spheroids, prompted us to investigate the impact of CRH signaling on the cytoarchitecture of more complex systems that closely mimic the human brain. In pursuit of this, we generated human cortical organoids, and the results paralleled those observed in the neural spheroids. Specifically, measurements of the ventricular zone revealed an initially larger size during early corticogenesis in CRH- and NBI-treated organoids, indicating a higher proliferative rate compared to the control. However, this phenotype was reversed later in development, providing additional support to our earlier findings that disrupted CRH signaling leads to accelerated differentiation of neural stem cells.

To unravel the molecular mechanisms behind these observed phenotypes, we conducted bulk RNA sequencing analysis to explore gene-level alterations. The Retinoic Acid (RA) metabolism pathway emerged as a major player affected by the manipulation of CRH signaling. Specifically, the expression levels of genes *CYP26A1* and *CYP26B1*, which encode the catabolic enzymes Cyp26a1 and Cyp26b1 responsible for Retinoic Acid (RA) degradation(Hernandez *et al.*, 2007; Ross and Zolfaghari, 2011), were found to be significantly reduced. This downregulation likely disrupts the normal degradation pathway of RA, potentially leading to elevated concentrations of Retinoic acid in instances of perturbed CRH signaling. During embryonic development, it is widely believed that the primary function of the Cyp26 enzymes is to regulate the RA synthesized from vitamin A, thereby establishing gradients of RA concentration. These gradients play a pivotal role in regulating the spatial patterns of gene expression that orchestrate various aspects of development, including the *HOX* genes (McCaffery and Simons, 2007; Nolte, De Kumar and Krumlauf, 2019). The role of retinoic acid (RA) in activating Hox patterning genes is well-established, with genes situated at the 3' end of the complexes to exhibit heightened responsiveness to RA. Consequently, the presence of RA typically induces the expression of anterior HOX genes first (Shimeld, 1996; Nolte and Krumlauf, 2013). This regulatory function of retinoic acid provides insight into the observed upregulation and downregulation of *HOXB* genes as revealed by the RNA sequencing analysis that we conducted.

Previous research from our group indicated that CRH enhances the neurogenic potential of adult Neural Stem Cells (NSCs) in the mouse hippocampus by inhibiting the BMP4 signaling pathway (Koutmani et al., 2019). Another study demonstrated that CRHR1 functions involve the upregulation of a transcription factor, REST, through which CRH enhances the pool of neural stem cells (Kwon et al., 2023). The intricate role of CRH is evident, acting through diverse molecular pathways dependent on developmental stage, brain origin, and the specific experimental model employed (organoids, spheroids). Notably, studies in mutant mice with Retinoic Acid deficiency reinforce the regulatory role of retinoic acid in proper corticogenesis. Thus, RA deficiency results in impaired RG progenitors pool, prematurely produced neurons, which later deplete and finally lead to microcephaly (Haushalter *et al.*, 2017).

Another recent study, presents the significance of RA in neural development and function in the neocortex of humans, while it is involved in the molecular patterning of prefrontal and motor areas (Shibata *et al.*, 2021). In addition, RA plays a crucial role in maintaining the differentiated state of adult neurons. Disruption of RA signaling in adults has been linked to the degeneration of motor neurons, leading to motor neuron diseases (Guidato, Barrett and Guthrie, 2003; Liu, Laufer and Jessell, 2001). Furthermore, it is implicated in the development of Alzheimer's

disease and may contribute to the onset of Parkinson's disease (Maden, 2007). The crucial role of RA is not only limited to the initial patterning and development of the brain, but also extends to a broader role in maintaining homeostasis for normal brain function throughout life (Wołoszynowska-Fraser, Kouchmeshky and McCaffery, 2020). In our study, utilizing two independent models, we observed an impaired maturation and structural formation when CRH signaling is disrupted. This disruption not only affected the cellular distribution but also led to malformed cytoarchitecture. These findings align with the observed abnormalities in retinoic acid metabolism, highlighting the complex interplay between CRH signaling and retinoic acid in regulating essential processes for proper brain development and function.

In conclusion, CRH emerges as a crucial modulator, exerting its influence through an as-yet-undiscovered molecular pathway, ultimately disrupting retinoic acid (RA) metabolism and resulting in impaired corticogenesis. This novel insight proposes a regulatory role of CRH in the complex processes of brain development, pointing towards potential avenues for therapeutic interventions and further research to elucidate the underlying molecular mechanisms. The fact that only a few hormones have been described to affect neurogenesis in fetal life depicts CRH as an interesting pharmacological target for potent therapeutic approaches of neurodevelopmental diseases (Buntwal et al., 2019; Hardeland, 2022; Schoenfeld and Swanson, 2021; Torner, 2016). Utilization of this innovative methodology, coupled with a deeper exploration of stress hormone interactions, holds significant promise for advancing our comprehension of neural processes and developing targeted interventions for disorders affecting neurodevelopment.

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Research Proposal

Excess maternal glucocorticoid exposure on fetal brain development

Project Summary:

Glucocorticoids (GCs) are a class of corticosteroids, which are class of steroid hormones produced from the cortex of the adrenal glands. Typically, GCs are released in response to stress or illness and play a pivotal role in regulating various biological processes, including inflammatory and immune responses, metabolic homeostasis, cognitive function, reproduction, and development (Chatuphonprasert, Jarukamjorn and Ellinger, 2018).

It is crucial to highlight the significant role of GCs during pregnancy and fetal development. Their influence extends beyond late gestation for lung maturation, encompassing a pivotal role in early gestation for successful fetal implantation (Busada and Cidlowski, 2017). Normal levels of GCs are tightly regulated by the placenta, acting as a protective barrier that filters maternal GCs, preserving the fetal compartment. However, perturbation of the mothers' levels of GCs has been linked to adverse outcomes in fetal brain development (Uno et al. 1994, Matthews 2000) and development of psychopathology (Schlotz and Phillips, 2009).

Given the essential application of antenatal steroids in cases of maternal pathological conditions or preterm births to facilitate lung maturation, optimizing treatment becomes paramount as corticosteroids, due to their ability to cross both the placenta and the blood-brain barrier (Crowther and Harding, 2016; Reynolds and Seckl, 2012), are implicated in causing mental and behavioral disorders in children (Mcdougall *et al.*, 2023). To address this knowledge gap, we propose as a research model the use of human cerebral organoids derived from human embryonic stem cells. This is a valuable model for studying fetal brain development and it will allow us to gain insight into the mechanisms underlying the impact of elevated glucocorticoid concentrations. In this context, both natural GCs like cortisol and synthetic ones such as dexamethasone will be systematically tested in terms of dosage and timing. Thus, GCs toxicity will be evaluated in a time window mimicking the physiological brain development and potent molecular, cellular, and structural abnormalities will be assessed. Additionally, stem cell cultures derived from both female and male sources will be employed to explore potential sex-specific effects of prenatal glucocorticoids on brain development.

Specific aims

The primary objective of this study is to investigate the consequences of abnormal concentrations of glucocorticoids on fetal brain development, exploring potential sex-related differences and determining therapeutic ranges for glucocorticoids (GCs).

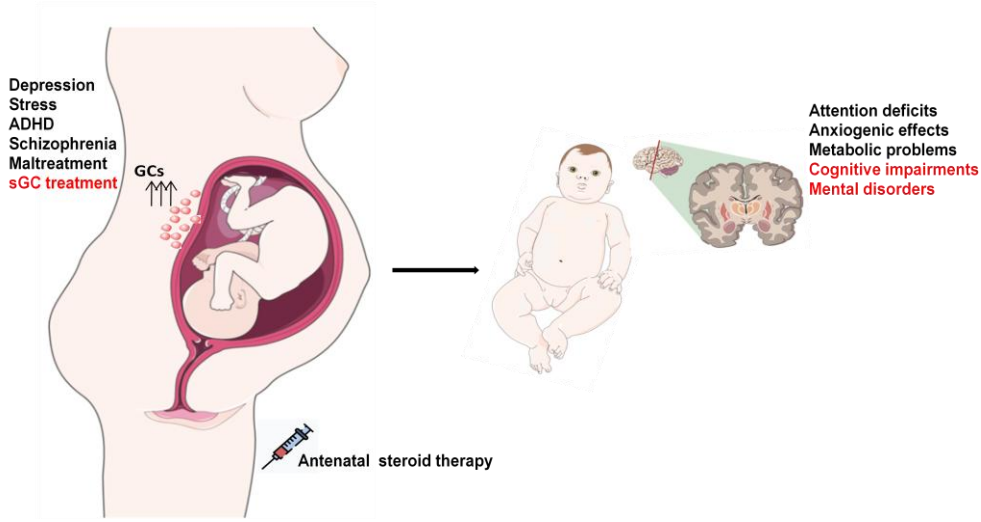
Unraveling the underlying molecular and cellular mechanisms of neurodevelopmental diseases has proven challenging, despite ongoing efforts in targeted drug discovery. Additionally, limitations arising from the inaccessibility of human brain tissue as well as the limited ability to accurately reproduce key features of human disease in animal models, further delay the development of therapeutic strategies. Leveraging experimental approaches, particularly the use of human cortical organoids, offers a humanized model for comprehensive study.

To identify the effects of disrupted GC levels on brain development, human cortical organoids will undergo treatment with varying concentrations of GCs within a time frame mirroring neurogenesis, facilitating structural and molecular analyses. A control group with physiological GC levels, based on existing literature, will be included for comparative analysis. This research aims to elucidate: (a) the developmental impact resulting from embryonic exposure to elevated GCs during pregnancy, (b) the molecular mechanisms through which increased glucocorticoid levels alter neurodevelopment, (c) the optimal therapeutic doses of antenatal GCs, and (d) molecular targets for potential drug development.

To achieve these objectives, an array of bioinformatics, genetic, and molecular techniques will be applied. High-content imaging (HC) will be utilized for the immunohistochemical analysis of organoid tissues, enabling the study of cortical structure and architecture using specific markers. Western blot analysis will provide insights into protein expression differences at the translational level. Coupled with proteomics analysis, RNA sequencing will offer comprehensive information at the transcriptomic level, aiding in the identification of novel molecular targets of GC action. Subsequently, highly expressed genes associated with an impact on neurogenesis, as identified through RNA sequencing, will undergo a PheWAS analysis to identify their effects on 7,323 phenotypes from the UK Biobank and the NHGRI-EBI GWAS Catalog. These phenotypes include, among others, neurobehavioral traits and adult neuroimaging data. The objective of this approach is to uncover the consequences of glucocorticoid overexposure on mental health, cognition, and brain structure. Ultimately, the aim is to formulate a theory that can be implemented for preventive measures.

Introduction and significance

Prenatal development is a critical determinant of health outcomes after birth. Disruptions in environmental factors, such as exposure to nutrient restriction, glucocorticoids or synthetic glucocorticoids, drugs, and stress, have been associated with a range of postnatal consequences affecting metabolic, cardiovascular, neurobehavioral, and brain structural aspects (Schmidt et al. 2018, Monk; Lugo-Candelas, and Trumpff 2019). Depending on the developmental stage of specific brain areas and circuits, exposure to prenatal stimuli determines the health status of the offspring. The central nervous system (CNS), characterized by a higher form of plasticity, undergoes substantial changes during development. Consequently, the intensity, timing, and duration of adverse environmental stimuli, along with early life stress (ELS), serve as major risk factors for the development of behavioral dysfunctions and mental disorders later in life (Bock *et al.*, 2014).



Pregnancy constitutes a period where both the fetus and the mother undergo vast changes, making this period highly sensitive to any stimuli. One of the key systems undergoing substantial changes is the hypothalamic-pituitary-adrenal (HPA) axis, which plays a crucial role in producing steroid hormones vital for the maturation and function of numerous organs. Under stress conditions, the hypothalamus releases corticotropin-releasing hormone (CRH), and the posterior pituitary gland releases vasopressin (AVP). These hormones, in turn, bind to receptors in the anterior pituitary gland, triggering the secretion of adrenocorticotrophic hormone (ACTH) into the bloodstream. ACTH, in sequence, stimulates the production of glucocorticoids. The latter, in a feedback loop, bind to steroid receptors expressed in the brain, leading to the termination of HPA axis activation and the secretion of glucocorticoids (Smith and Vale, 2006). During pregnancy, the placenta also secretes CRH in both the maternal and fetal compartments. In contrast to the negative feedback exerted by glucocorticoids in the hypothalamus, maternal and fetal glucocorticoids during pregnancy actually enhance the production of CRH from the placenta (O'Donnell, O'Connor and Glover, 2009)

At the end of gestation, elevated CRH and GCs levels play a pivotal role not only in the initiation of parturition but also in various other physiological functions. These functions include supporting the mother's energy needs through hepatic gluconeogenesis and the suppression of inflammation (Munck, Guyre and Holbrook, 1984). In addition, GCs promote fetal lung maturation (Harding *et al.*, 2012) and it has been shown to be implicated in the brain development by affecting, among others, neuron to neuron and neuron to glia interactions (Matthews, 2000) and synaptic properties (Lupien *et al.*, 2002).

These massive changes in maternal and fetal HPA axis during pregnancy highlight the vital role

of GCs for the physiological development of the fetus. Consequently, these functions imply that deviations from the physiological range of GCs could potentially disrupt fetal development and exert profound effects in postnatal life.

While it is well-established that pregnancy represents a sensitive period where prenatal environmental exposures significantly impact neurobehavioral and physiological outcomes in offspring after birth, the precise mechanisms through which glucocorticoid treatments or elevated maternal levels mediate these effects remain less understood. Previous studies have indicated that both excess endogenous glucocorticoid exposure *in utero* (resulting from stress) and exogenous glucocorticoid exposure through pharmacological treatment can reduce birth weight and alter the sensitivity of the HPA axis (Rodriguez et al. 2019; Moisiadis and Matthews 2014). However, the molecular and genetic mechanisms behind these abnormalities as well as the structural and cellular modifications in fetal brain remain unexplored. Given the indispensable and often unavoidable nature of elevated GC levels during pregnancy, understanding their effects on fetal brain development and determining therapeutic ranges with minimal risks becomes mandatory.

To address this knowledge gap, a 3D in vitro system simulating brain development will be examined under conditions of elevated glucocorticoids at different time-windows. It is anticipated that subjecting cortical organoids to GC exposure will unveil the mechanisms underpinning the effects of corticosteroids, shedding light on their distinct roles in human physiology. This exploration holds promising avenues for pharmacological applications in neurodevelopmental disorders and anxiety-related diseases.

Research strategy

A more comprehensive understanding of the pathomechanisms involving glucocorticoids during pregnancy, leading to stress-related psychiatric disorders in offspring, is crucial for the development of more effective preventive and therapeutic strategies. To date, data on the impacts of prenatal exposure to glucocorticoids have primarily been derived from clinical studies, often assessed only after the manifestation of symptoms in the offspring, thus leaving a gap in early diagnosis. Furthermore, animal studies have been conducted, revealing that prenatal exposure to glucocorticoids results in altered HPA axis activity, anxiety, and schizo-affective disorders (Welberg, Seckl, and Holmes 2001; Tronche et al. 1999)

However, cellular properties such as neural stem cell proliferation, neurogenesis, migration, and maturation display species-specific characteristics, leading to variations between mice and

humans. Notably, a significant distinction lies in the human-specific expression of placental CRH, which may play a crucial role in brain development in anthropoid primates (Kassotaki et al. 2021; Power and Schulkin 2006). Consequently, the study of prenatal effects of glucocorticoids in mouse models, which exhibit such divergences, becomes impractical. Additionally, due to ethical and practical constraints, obtaining human brain samples for experimental purposes proves challenging.

This study aims to enhance our comprehension of the impact of prenatal glucocorticoids (GCs) on the development of psychopathologies in offspring. To achieve this, we employ an in vitro model designed to mimic human embryonic development.

The objectives that are going to be addressed are the following:

1. How elevated concentrations of GCs affect the size, structure and architecture of cortical organoids

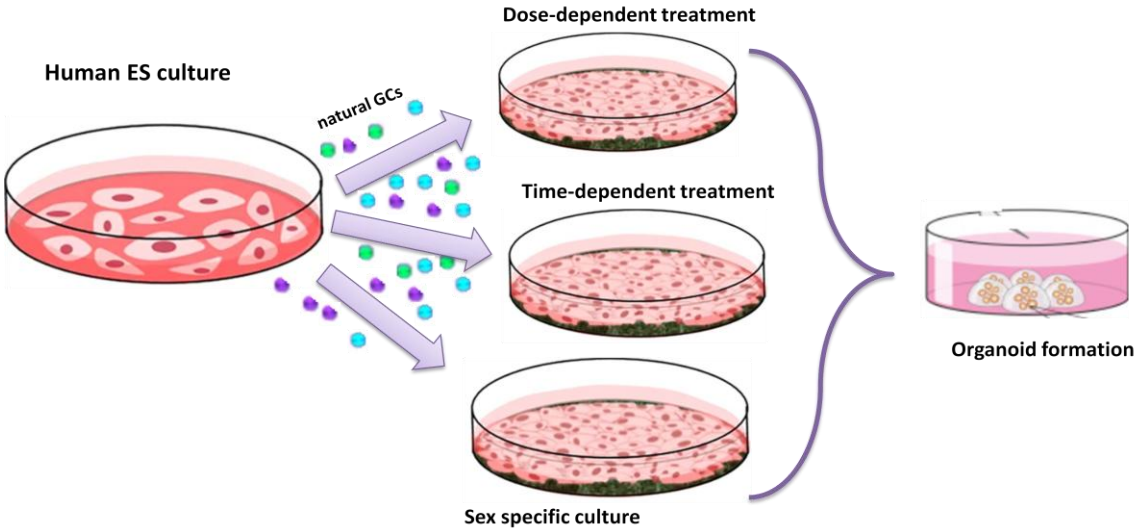
2. What are the molecular and genetic pathways involved in the GC action on brain development

3. Do GC act in a sex related way?

Work package (WP)

For this experiment, two cortical organoid models will be generated, originating from cells of different gender origins, to uncover potential sex-dependent variations. Both models will undergo identical treatment, being exposed to various glucocorticoid concentrations at different time points, mirroring those observed in the steroid treatment of pregnant women. The primary objective is to identify molecular pathways induced by glucocorticoids that impact the developing neural system.

To achieve this, cortical organoids will undergo glucocorticoid treatment at distinct developmental stages and will be subject to independent immunohistochemical and genetic analyses at each time point. At the end of the experiment, a comparative analysis of the cytoarchitecture and gene expression of the organoids at each time point, in comparison to an untreated control group, will be conducted. Sex-specific differences will also be assessed.



To extract comprehensive information about the various cell types present in the developing brain, specific markers of the neural lineage, such as GFAP for astrocytes, Nestin for neural stem cells, Olig2 for oligodendrocytes, beta-tubulin III for neurons, vGLut for glutaminergic neurons, and GAD for GABAergic neurons, will be utilized. Additionally, detailed insights into the structure and cytoarchitecture of the 3D developing cortical organoids will be provided through the application of layer-specific markers such as TBR1, CTiP2, SATB2, CUX1, and Reelin (Molnár *et al.*, 2019).

In addition, the study will include the examination of gene expression patterns through single-cell RNA sequencing, offering valuable insights for targeted therapy. This analysis aims to identify new molecular pathways adversely affected by glucocorticoid treatment (Cheng *et al.*, 2016). Subsequently, a PheWAS analysis will be conducted to identify the effects of these pathways on children's phenotypes (Bastarache, Denny and Roden, 2022). In conclusion, the collective findings from these analyses will not only unravel the molecular mechanisms underlying by the excess maternal glucocorticoid exposure for preventive measures but will also uncover novel targets for future drug development.

BUDGET		
Category		**Total in €**
Direct Costs Personnel		
Post-Doc Researcher (12 months)		30,000
Research Staff Scientist (12 months)		20,000
Total Direct costs for Personnel		50,000
Other Direct Costs	**Justification**	
6.1.2 Consumables	Stem cells, feeders, antibodies, enzymes, plasticware, special kits, special consumables, Cell culture media, etc	100,000
6.1.3 Dissemination	Participation in international meetings, publication fees etc	15,000
6.1.4 Use and/or Access to equipment etc.	Single-cell RNA sequencing	40,000
6.1.5 Equipment	Small-scale lab equipment, computers etc	10,000
6.1.6 Other Costs	Single-cell RNA sequencing analysis	5,000
6.1.7 Purchase of animals		-
Total "other direct costs"		170,000
Total Direct Costs		50,000
Indirect Costs (Institution overhead, 10%)		22,000
Total Budget		222,000

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Curriculum vitae

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WORK EXPERIENCE

2022/02-current — **Laboratory Administrator of molecular genetics laboratory,** Medicon Hellas, Athens, Greece

- Data analysis and assay examination
- Supervising the department of molecular biology's adherence to Good Laboratory Practice (GLP).

2019/09-2021/12 — **Clinical Laboratory Scientist**, Athens Lab, Athens

- Manager responsibilities: review laboratory protocols, quality testing to ensure equipment performance, training and supervision of laboratory staff, responsible for overseeing safety and security of laboratory facilities

EDUCATION

2021/10-2024/05 — **Master of Science (MSc) : Athens International Master's Programme in Neurosciences**
NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS, Athens, Greece

2014/10 – 2019/06 — **Bachelor of Science: Molecular Biology And Genetics**
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS - DEMOCRITUS UNIVERSITY OF THRACE, Alexandroupoli, Greece

SKILLS

Computer skills

ECDL Foundation - ICDL - International Computer Driving Licence : advanced user

- MS Windows
- MS Office (Word, Excel,PowerPoint, OneNote)
- GraphPad Prism 8
- Mendeley
- NCBI tools (PubMed, BLAST, CLUSTAL)

Languages

English: Examination for the Certificate of Proficiency in English (ECPE),
IELTS Overall Band Score: 7

German: Elementary Level

Greek: native speaker

Other skills: communicative with a team spirit and interpersonal skills, hard-working, organizing

Driving Licence: B