HELLENIC REPUBLIC

National and Kapodistrian University of Athens

Department of Biology

Athens International Master's Programme in Neurosciences

1st DEPARTMENT OF PHYCHIATRY, NKUA

UNIVERSITY MENTAL HEALTH RESEARCH INSTITUTE

RESEARCH THESIS PROJECT

IMMUNOHISTOCHEMICAL STUDY OF THE GLUCOCORTICOID RECEPTOR IN THE LOCUS COERULEUS OF HUMAN NEONATES: POSSIBLE ROLE OF PERINATAL HYPOXIA

Foteini Dareioti, ID: 111709

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Three Member Evaluation Committee	**Signatures**
Prof. Maria Panayotacopoulou (supervisor)	
Prof. Efthimia Kitraki	
Associate Prof. Antonios Stamatakis	

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To my parents and my brother,
for their love and their support.

Contents

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Foteini Dareioti

Title

Immunohistochemical study of the glucocorticoid receptor (GR) in the locus coeruleus of human neonate: possible role of perinatal hypoxia.

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Summary

Early life stress, as hypoxia, during the critical perinatal period may play a role in the onset of both somatic and neuropsychiatric disorders later in life. Locus coeruleus (LC) participates in stress response increasing norepinephrine release. Cortisol, the main mediator of stress response, acts via mineralocorticoid (MR) and glucocorticoid (GR) receptors. In the brain, GR is abundant in hippocampus and LC. In the present study, we immunohistochemically investigated the effect of perinatal hypoxia on GR expression in the LC neurons of fetal/neonatal human brains with neuropathological lesions of acute or prolonged hypoxia. Our results showed that in full-term neonates, GR immunoreactivity in the nucleus of LC neurons was negatively correlated with hypoxia grading. Low GR immunoreactivity was observed in the nuclei of LC neurons in stillborn embryos. Based on our observations, we suggest that changes in GR expression due to perinatal hypoxia may predispose the survived infants to stress-related disorders.

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Highlights

- In full-term infants, GR immunoreactivity in the nucleus of noradrenergic LC neurons exhibited negative correlation with hypoxia grading.
- Stillborn embryos had lower GR immunoreactivity in the nucleus of noradrenergic LC neurons compared with the live-born ones.
- Cellular and nuclear size of LC neurons was decreased in prolonged hypoxia.

Keywords

Glucocorticoid receptor, perinatal hypoxia, early life stress, human neonates, immunohistochemistry, Locus coeruleus, tyrosine hydroxylase, cortisol, noradrenaline, stillbirth

Introduction

During the last decades, it has been widely accepted that events taking place within a special time window of development play a crucial role in the possibility of emergence of various somatic and neuropsychiatric diseases in later life (Barker, 2003), (Taylor, 2010), (Kara and Sherin, 2011),(Li et al., 2012). Such a time window is perinatal period, starting at 22 completed weeks of gestation and lasting through seven days after birth (Nguyen and Wilcox, 2005). Stressful stimuli exerted on embryo/neonate along this time - called early life stress - contribute to the high risk for several neurological and psychiatric disorders (Rees et al., 2011). The mechanism underling the fore mentioned phenomenon consists of epigenetic alterations influencing gene expression (Mpofana et al., 2016) (Faa et al., 2016).

A very common type of physical early life stressors is hypoxic/ischemic injury (HII) during perinatal period. Perinatal hypoxia-ischemia (PHI) is a causative and very frequent factor for embryo/neonate's death and seems to be responsible for a variety of malfunctions in the brain of offspring survived. The most studied clinical condition of perinatal HI is hypoxic-ischemic encephalopathy (HIE) due to the severity of its symptoms (Antonucci et al., 2014) (Millar et al., 2017). Epidemiological studies in developed countries have shown that HIE appears an incidence of 2 – 6 and 7 in every 1000 live full-term and pre-term births, respectively. Of infants experienced HIE, 15 -25 % will pass away within postnatal period. A proportion around 25 % of neonates survived from PHI will come out with enduring serious long-term impairments (Edwards et al., 2018). The deficits belong to a large spectrum of neurological and psychiatric malfunctions including dyskinetic tetraplegic cerebral palsy, epilepsy and seizures, learning, cognitive and mental impairments, vision impairments, general developmental retardation as well as motor and behavioral problems (Riljak et al., 2016), (Lai and Yang, 2010). Furthermore, strong evidence indicates a link between perinatal hypoxia and increased risk of development ADHD, autism spectrum disorder, schizophrenia and Parkinson's disease even in the absence of functional motor disorders (Nalivaeva et al., 2018), (Hefter et al., 2018),(Faa et al., 2016), (Miguel et al., 2015), (Zhu et al., 2016), (Giannopoulou et al., 2018), (Pagida et al., 2013).

The two major systems activated in stress response are the Sympathetic Nervous System (SNS) and the Hypothalamic-Pituitary-Adrenal Axis (HPA axis). The effect of the first one is composed of the adrenal medulla stimulation leading to the release of epinephrine and norepinephrine into the blood and thus, switching on the "fight or flight" response. Regarding the second system, hypothalamus secretes corticotropin-releasing factor (CRH), which triggers the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal cortex to release cortisol, a hormone affecting crowd of functions like immune reaction,

metabolism, skeletal growth, cardiovascular function, reproduction, and cognition (Taylor, 2010), (Oakley and Cidlowski, 2013).

Cortisol acts through mineralocorticoid (MR) and glucocorticoid receptor (GR). GR has lower affinity for cortisol than the MR (Gomez-Sanchez and Gomez-Sanchez, 2014). GR gene *NR3C1* (nuclear receptor subfamily 3, group C, member 1) is located in chromosome 5 and contains 9 exons, which through different splicing give rise to different variants. GRα is the typical and most abundant variant of GRs and mediates the glucocorticoids' actions. GRβ does not bind glucocorticoids and it seems to serve as negative regulator of GRα, whereas GRγ has similar profile to GRα, but it is inefficient in regulating glucocorticoid responsive genes. In addition, there are lots of different translational isoforms of GRα regarding the initiation point of translation onto the mRNA as well as plenty of post-translational modifications that enhance the variety of GRs (Oakley and Cidlowski, 2013), (Kadmiel and Cidlowski, 2013). GR performs two mechanisms of action: the genomic and the non-genomic. Starting with the genomic one, GR is a cytoplasmic protein in the absence of ligand. When GR binds glucocorticoids, inserts the nucleus and there is dimerized. The dimer interacts with the GRE (glucocorticoid responsive elements) on the DNA sequence and as a transcription factor alters the expression of different genes. Concerning the non-genomic mechanism, GR is bound to the cell membrane and coupled with G-proteins. Thus, its activation results in a cascade of downstream proteins' interplays (Kadmiel and Cidlowski, 2013), (Oakley and Cidlowski, 2013), (Scheschowitsch et al., 2017). Nevertheless, another way of action has been reported, the genomic mechanism of action in mtDNA. GR enters mitochondria's stroma and modulates metabolic and apoptotic processes through regulation of mitochondrial genes (Du et al., 2010), (Sionov et al., 2006). GR has a ubiquitous expression in every kind of cell or tissue, except for red blood cells (Scheschowitsch et al., 2017). In the human brain, hippocampal formation and amygdala are characteristic areas of high GR expression (Wang et al., 2013), (Wang et al., 2014). Studies in rats investigating the GR distribution all over the brain have shown that Locus coeruleus exhibits also high GR levels, equal to hippocampus (Morimoto et al., 1996), (Sánchez et al., 2000).

Locus coeruleus (LC) is a nucleus of norepinephrine-containing neurons, found the in upper dorsolateral pontine tegmentum, in the lateral aspect of the fourth ventricle (Benarroch, 2009), (Pagida et al., 2016). LC neurons project to the whole brain and spinal cord and thus, play crucial role in a plethora of functions, such as in sleep and wakefulness, memory, attention and learning, emotion, reproduction, and responses of the central nervous system to stress (Samuels and Szabadi, 2008), (Itoi and Sugimoto, 2010). Specifically, LC is considered to receive excitatory input from CRF-producing areas (hypothalamic PVN, amygdala, limbic cortex) as well as to send excitatory output to the same areas forming a positive loop (Valentino and Van Bockstaele, 2008), (Atzori et al., 2016). According to the above, norepinephrine neurons of LC produce high amounts of GR protein and several surveys proved that GR-ligand complex could modulate the expression of Tyrosine hydroxylase (TH), the

first enzyme in the process of norepinephrine's synthesis, in these neurons (Markey et al., 1982), (Makino et al., 2002), (Sukhareva et al., 2017). However, the results are not always in symphony and differ depending on the dose, the period of the exposure to glucocorticoids and the animal strain or the cell line type. A proposed mechanism for the dialogue between glucocorticoids and TH is based on the interaction of activated GR with other transcription factors, especially AP-1 (Sukhareva et al., 2017).

The purpose of the present study is to investigate the GR immunoreactivity in the LC of the human neonate and the possible range of GR expression between the different hypoxic conditions (duration and severity of hypoxic episode) trying to add a brick into the broader effort for understanding the role of perinatal hypoxia as an early life stressor in the development of neurological and psychiatric disorders later in life.

Materials and Methods

Patients, Tissues and Histopathology

The material used in the present study consists of formalin-fixed infant autopsied brains, obtained from the Greek Brain Bank (GBB; member of Brain-Net Europe, directed by Professor E. Patsouris). Complete postmortem examination was performed in all cases after parental written consent for diagnostic and research purposes. Part of the material was used in our previous study (Pagida et al., 2016). In total, 21 infant brains were studied, 12 male and 9 female. Six of them (GBB 1836/06, 2062/07, 1286/04, 1402/04, 276/17 and 170/16) were born prematurely before 37 week of gestation, while the remaining neonates were delivered at or near term. The corrected neonatal age (duration of pregnancy + postnatal age) was ranged from 32 to 46 weeks. Most subjects were liveborn apart from the following: 2631/09, 3340/15, 170/16, 311/18, 3161/13 who were either fresh stillbirths or intrapartum deaths. The clinical and pathological data regarding the material are presented in the Table 1.

The neuropathological evaluation of neonatal hypoxic-ischemic encephalopathy was relied on established criteria based on the pattern of gray and/or white matter lesions (included in the spectrum of neuronal necrosis and periventricular leukomalacia changes) in the frontal and occipital cortex, basal ganglia, thalami, and brainstem (Table 2). Notably these criteria involve morphological alterations that is, cellular pyknosis or cytoplasmic swelling with chromatolysis in mature neurons, karyorrhexis, and focal, laminar, or diffuse loss of neurons in the cortical layers. The grading of the duration and the severity of hypoxic injury lean on the forementioned benchmarks taking into account the regional aspects of neuronal necrosis. Thus, three neuropathological groups of HII arise: group 1 consistent with severe/acute HII; group 2 consistent with moderate/prolonged or older injury, and group 3 consistent with very severe/long duration or chronic HII. When multiple lesions

coexisted (combinations of gray and white matter injury or multiple lesions of differing ages or severity), the highest score observed was assigned to the case (Pagida et al., 2013), (Pagida et al., 2016).

In view of the limitation of working with human autopsy samples and considering that all autopsied neonates who fulfilled the criteria of inclusion sustained some degree of hypoxic insult, true 'controls' deprived of any sign of histopathological hypoxic injury could not be included in the present study. Case GBB 170/16 has been classified as grade 0 since neuropathological examination did not showed any signs of neuropathological injury in the specific brain areas analyzed above. Thus, it was used as control. As positive control of the GR staining, hippocampal paraffin section of 1 adult subject (B11) was added from our archival collection, a 62-year-old male who died from coronary artery occlusion (postmortem delay 23 hours; autolysis time: 23 hours, fixation time: 16 hours in 10% normal buffered formalin) (obtained from the archival M. Issidorides brain tissue collection of Eginition Hospital, University of Athens), as in the literature such tissues give a positive reaction for GR (Wang et al., 2012, 2013). We, also, used sections of a fetal hippocampus (GBB 2631/09), as our samples consist of fetal material.

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GBB No.	Age (w, d, h)	Total age, (w)	Sex	Postmortem delay (days)	Fixation time (months)	Body Weight		Brain Weight (g)	Head Perimeter		Clinical and pathological data[a]/ Medications	HII Group
						(g)	percentile		(cm)	percentile		
2807/07	37w	37	M	2	2.5	2445	10-50	444	32.5	10-50	Acute thrombosis of the umbilical vein	1
2631/09	38w	38	F	<0.5	3	3970	90-97	392	36.5	90-97	Stillborn infant of diabetic mother – Macrosomia, Cardiomyopathy, Organomegaly, Pancreatic islet hyperplasia	1
1705/05	37w+8d	38	M	2	2	2600	10	345	32.5	10-50	Genetic thrombophilia, thrombosis of the descending aorta, thrombotic vasculitis, meconium aspiration, hypotension, hyperglycaemia / Antibiotics, adrenaline, dopamine, inotropes, TPN	2
1965/06	39w+2h	39	M	2	1	2744	3-10	337	34.0	10-50	Congenital cyanotic heart defect/Adrenaline, bicarbonates	2
2735/09	39w+2d	39.5	F	0.3	6	2960	10-50	313	32.0	3	Fatty acid oxidation defect – Liver steatosis, Cardiomyopathy, Pancreatic islet hyperplasia	1
1836/06	35w+29d	39	M	3	8	1950	<3	310	31.0	<3	Ivemark syndrome (asplenia – congenital cyanotic heart defect – heterotaxy) / Antibiotics, inotropes, adrenaline, TPN	2
3907/07	39.5w+2h	39.5	F	1.5	1	3255	10-50	380	35.0	50-90	Lung atelectasis /Adrenaline, bicarbonates	1
1593/05	41w+1d	41	M	2	2	3120	3-10	380	33.0	3-10	Congenital cyanotic heart defect, congenital viral infection, meconium aspiration /Antibiotics, inotropes, adrenaline	3
1846/06	37w+34d	42	F	1	3	ND	ND	635	40.0	>97	Brochopneumonia, reactive hepatitis	1
2062/07	28w+103d	43	M	0.7	4	2280	<3	283	30.5	<3	Cystic fibrosis, RDS, respiratory infection /Surfactant, antibiotics, inotropes, diuretics, sedatives, TPN	3

1286/04	35w+67d	44.5	M	3	12	3800	3-10	347	34.5	<3	Placental insufficiency, Respiratory infection, Cholestasis, Adrenal hypoplasia /Antibiotics, anticonvulsants, sedatives, inotropes, adrenalin, TPN	3
1402/04	25w+136d	44	M	3	8	3000	<3	300	34.0	3	RDS, renal failure, congenital cystic renal hypodysplasia, endocardial fibroelastosis, myocardial ischemia /surfactant, antibiotics, corticosteroids, anticonvulsants, inotropes, diuretics, sedatives, TPN	3
2325/07	39w+49d	46	F	0.4	6.6	2890	<3	413	33.5	<3	RDS, Genetic surfactant C deficiency /Surfactant, antibiotics, sedatives, inotropes, TPN	3
3161/13 (2012)	40w	40	F	1	1.4	3100	10-50	467	34.5	10-50	Intrapartum death: Umbilical cord prolapse, hypoxic placenta / (-)	1
3340/15	39.5w	39.5	F	4	1.1	2760	~10	416	32	3	Stillborn: occlusion of omphalic vein, thrombotic occlusion; encapsulated findings incompatible with cytomegalovirus not confirmed. (hypoxia 2-3 days with a critical phase 6-12 hours before death)	2
276/17	32w+11h	32	M	3	4.7	2078	50-90	279	32.8	>97	Respiratory failure; neurogenic myopathy	2
170/16	35w	35	F	2	12	3493	>97	348	34.7	>97	Stillborn: maternal diabetes, acute hypoxia, villous immaturity	0
3415/16	37.5w + 0.5h	37.5	F	3	23	3213	50-90	416	34.5	50-90	Gongenital pneumonic hypoplasia ; Chondroectodermal dysplasia (ellis van creveld); acute limited hypoxia	1
237/17	39w + 2d	39	M	1	2	3030	10-50	367	34.5	50	Gongenital inflammation of lungs, Ventriculomegaly; intrauterine neurotrophic infection), hypoxia 6-12 hours before death	2
235/17	40w + 3h	40	M	1	2.5	3474	10-50	384	33.8	10-50	Irreversible cyanotic cardiovascular abnormality	2

311/18	40w	40	M	2	2	3330	10 -50	495	36.8	~90	Stillborn: Maternal-Embryonic Bleeding / Transfusion (Anemia)	2

Features in bold are consistent with neonatal hypoxic/ischemic encephalopathy. w = Weeks of gestation; h or d = hours or days of postnatal life; RDS = respiratory distress syndrome; TPN = total parenteral nutrition; ND = not determined

[a] Excluding neuropathological findings.

Table 2. HII groups based on neuropathological criteria

HII			
Gray matter injury	Severe, abrupt	Moderate, prolonged, older	Very severe, long duration, old
Topography of neuronal necrosis	Thalamus, basal ganglia	Cerebral cortex, thalamus, basal ganglia	Diffuse neuronal necrosis, neuronal mineralization
White matter injury	Acute	Subacute	Chronic
Histopathological findings	Coagulation necrosis**,** axonal swellir	Endothelial hyperplasia, microglial proliferation, micro-calcifications, reactive gliosis	Glial scar, cavitation
HII groups	Group 1	Group 2	Group 3

Histology and Immunohistochemistry

Tissue parts of the pons at the level of LC were isolated and fixed in 10% formalin. After washing, tissues were dehydrated in graded alcohol, xylene and embedded in paraffin. The paraffin blocks were cut in 7-micrometer-thick serial sections along the entire rostrocaudal extent of the LC. One per 50 sections was mounted on silane-coated slide and stained with luxol fast blue/cresyl violet, as previously described (Marianna A. Pagida et al., 2013) in order to identify the boundaries of LC nucleus. Also, 1 section every 100, was stained with hematoxylin and eosin (HE) for routine histological evaluation an detection of possible necrotic and pyknotic neurons (Fischer et al., 2008).

Two successive sections at the caudal levels of LC (see fig. 42 in Paxinos and Huang (Paxinos and Xu-Feng, 1995), where the noradrenergic neurons are densely packed in a well-defined area of the ventromedial tip of the mesencephalic trigeminal tract, were immune-stained for TH and GR in order to estimate the total number of LC neurons and to disclose the number, distribution and intensity of GR reaction in LC neurons, respectively. For the detection of TH, sections were deparaffinized in xylene (2 × 10 min), rinsed in graded alcohol and washed in distilled water (2 × 5 min). Afterwards, they were placed in a plastic jar with citrate buffer (0.01 M, pH 6.0) and entered in microwave (2 × 5 min, 400 W) to unmask buried antigen positions. Subsequently, they stayed 20 min at room temperature (RT) for cooling, were washed with distilled water (3 × 2 min) and TBS 1x (pH 7.6) (2 × 5 min) and incubated in 5 % dry milk in TBS 1x for 45 min at RT in order to block non-specific antibody binding. After rinsing in TBS 1x (2 × 5 min), sections were incubated in 5 % normal goat serum (NGS, Vector Laboratories S-1000, USA) diluted in TBS 1x (30 min, RT) for further non-specific antigenicity restriction and incubated in antibody serum for TH (1: 1,000 in TBS 1x, polyclonal/rabbit (Millipore, AB152, USA)) for 1 h and then, overnight at 4° C. Next day, sections were washed in TBS 1x (3 × 5 min) and incubated in a secondary polyclonal goat anti-rabbit immunoglobulin/HRP antibody (1: 100 in TBS 1x, Biotinylated Goat anti-rabbit, Vector Laboratories,BA-1000, USA), stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB, D5905 Sigma Aldrich, USA) containing nickel ammonium (0.02% Nickel ammonium sulphate, Johnson Matthey Alfa Products, Germany) for 15 min, dehydrated in ethanol solutions with increasing concentration, and then cleared in xylene (2 × 10 min) and mounted in DPX Mountant medium (BDH, Poole, UK).

For the detection of GR, a modified protocol from Wang et al. was followed (Wang et al., 2013). That special and time-consuming protocol includes double incubation almost in all immunohistochemical steps, as GR is a hidden epitope, difficult to be caught. The steps are described below: rehydrated sections passed the antigen retrieval step (citrate buffer 0.01 M, pH 6.0, microwave 4 × 3 min, 400 W) and cooled down (20 min, RT). Then, they were incubated in 0.3% H2O2 in distilled water (30 min in dark place) to block endogenous peroxidase activity, rinsed in TBS 1x (3 × 5 min) and incubated in the primary antiserum for GR (1:75 in supermix, 1:100 for fetal hippocampus, ED5, sc-56851, Santa Cruz Biotechnology, against a peptide mapping within the N-terminus of GR) for 1 hour at RT, followed by an overnight incubation at 4 °C. After washing with TBS 1x (3 × 5 min), secondary monoclonal biotinylated goat anti-mouse IgG (1:100 in supermix, Vector Laboratories, BA-9200, USA) was added

for 1 hour at RT. For amplification of the signal, the incubations in both primary and secondary antibody were repeated for 1h at RT with TBS washing in the meantime. Washing with TBS 1x (3 × 5 min) followed and incubation in a 1:800 dilution of streptavidin-biotin-peroxidase complex (ABC Elite Kit, PK-6100) in supermix for 1 hour at RT. After rinsing in TBS, tyramide (1:750 in TBS 1x, 0.01% H2O2; an offer from Dr I. Huitinga, NBB Amsterdam) was added (30 min, RT, darkness) for further intensification of the signal. Washed in TBS sections were incubated in 1:800 dilution of streptavidin-peroxidase complex in supermix for a second time (45 minutes at RT). Then the sections were rinsed in TBS 1x (3 × 5 min) and Tris-HCl 1x for 15 min and incubated in DAB-nickel solution at RT for 8 minutes, rinsed in tap water, dehydrated, cleared in xylene, and coverslipped with DPX. The same procedure was followed for the adult hippocampal section used as positive control.

Morphometric Analysis

For the morphometric analysis of the optical density (OD) for GR immunostaining as well as cell and nucleus size, images of all the noradrenergic neurons containing nucleus from all the cases were unilaterally captured (LC is bilaterally homogeneous) at a magnification of ×400 by a digital charge-coupled device color video-camera (SSC-C370P; Sony, Tokyo, Japan) connected to an optical microscope (BX50F-3; Olympus, Tokyo, Japan). The measurements of the size and OD for GR in both cytoplasm and nucleus were executed using the image analysis software Image-Pro Plus, version 4.5.1.29 (Media Cybernetics, Bethesda, Md., USA).

Statistical Analysis

Statistical analysis of morphometric data was performed by using the Spearman's rank correlation coefficient due to both small number of cases included and non-canonical distribution of variables studied. OD for GR immunostaining in cytoplasm and nucleus as well as cell and nucleus size were investigated with regard to the neuropathological hypoxia grading, the total corrected (gestational and postnatal) age, postmortem delay, fixation time, body and brain weights, head perimeter, sex, stillbirth, infection and maternal diabetes. $p < 0.05$ was considered statistically significant. All tests were performed using SPSS, version 18.0.0 (SPSS, Chicago, Ill, USA). Given that the maturation of organs - especially of the lungs - is linked to cortisol levels in fetus (Davis and Curt A., 2010) (Agnew et al., 2017), we thought that preterm and full-term infants may react differentially in stress (Martin et al., 2018). So, we divided samples into the following subgroups: preterm (gestational age <37 weeks) and full-term (gestational age >= 37 weeks) and we statistically examined all the possible correlations to investigate whether any factor affects GR expression separately in these two subgroups (Cha and Masho, 2013).

Results

Delineation of LC through TH staining

Serial sections were stained with LFB/CV and TH-immunohistochemistry in order to specifically detect the noradrenergic neurons of LC, which are both LFB/CV and TH positive, from other cell types, such as glial cells and interneurons, stained only with LFB/CV. The total number of cells located in LC are depicted in figure 1a while the TH-immunoreactive (TH-IR) cells are shown in figure 1b. Their morphology is indicated in higher magnification in figure 1c and 1d, respectively. A differential signal intensity among the cases was observed. However, the presence of TH was clear in all cases and hence, noradrenergic neurons were easily distinguished. TH was mainly expressed in the cytoplasm of neuronal bodies and their close processes (Fig. 1d).

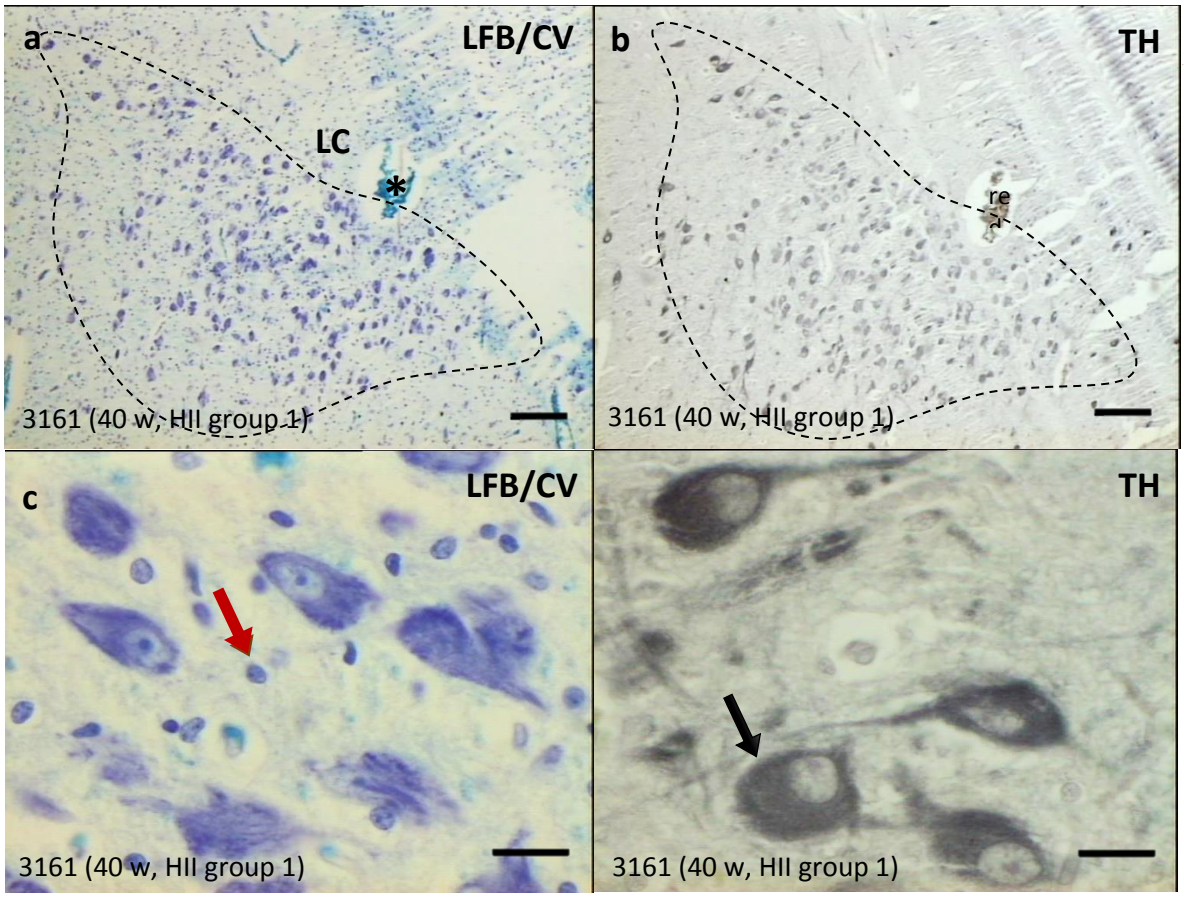

Figure 1.*Delineation of LC through TH staining.* Two adjacent sections at the caudal level of the LC of case GBB 3161/ 13 (40 weeks of age, HII group 1). In figures **a** and **b,** LC nucleus was stained with LFB/CV to delineate the borders of the nucleus and respectively with TH to reveal the TH-IR neurons. LC limits are marked with discontinuous line. In **c** and **d**, TH-IR neurons are shown in higher magnification, stained with LFB/CV (**c**) and TH (**d**). Note the intense staining of neuronal bodies and processes of LC neurons with TH-immunohistochemistry (black arrow). Also, note the glial cells stained with LFB/CV (red arrow). LC = Locus coeruleus, LFB/CV = luxol fast blue/cresyl violet, TH = tyrosine hydroxylase. Black asterisks show blood vessels in adjacent sections. Scale bar (**a**, **b**) = 150 μm, scale bar (**c**, **d**) = 20 μm.

GR-immunoreactivity in adult and fetal hippocampus (Standardization of the method)

Hippocampal sections of an adult human case (GBB B11) were used to standardize the immunohistochemical reaction for GR. In the adult human hippocampus, the signal was very clear and distinct in both nucleus and cytoplasm. In the dentate gyrus the nuclei of GR-immunoreactive neurons (GR-IR) were intensely stained in contrast to cornu ammonis (CA) areas where GR-immunoreactivity was mainly found in the cytoplasm, as it is shown in figure 2c. However, neurons with also clear nuclear distribution were found (Fig. 2b). Sections processed without incubation in GR antibody used to check the specificity of immunohistochemical reaction were free of staining. (Fig. 2d).

In the fetal hippocampus, the signal was clear in both cytoplasm and nucleus. Specifically, in the dentate gyrus the nuclei of neurons exhibited strong signal for GR, as it is depicted in figure 2e. In the whole area of CA, GR-immunoreactivity was mainly detected in the cytoplasm of neurons (Fig. 2f), but a percentage of neurons with intense nuclear signal was observed, too.

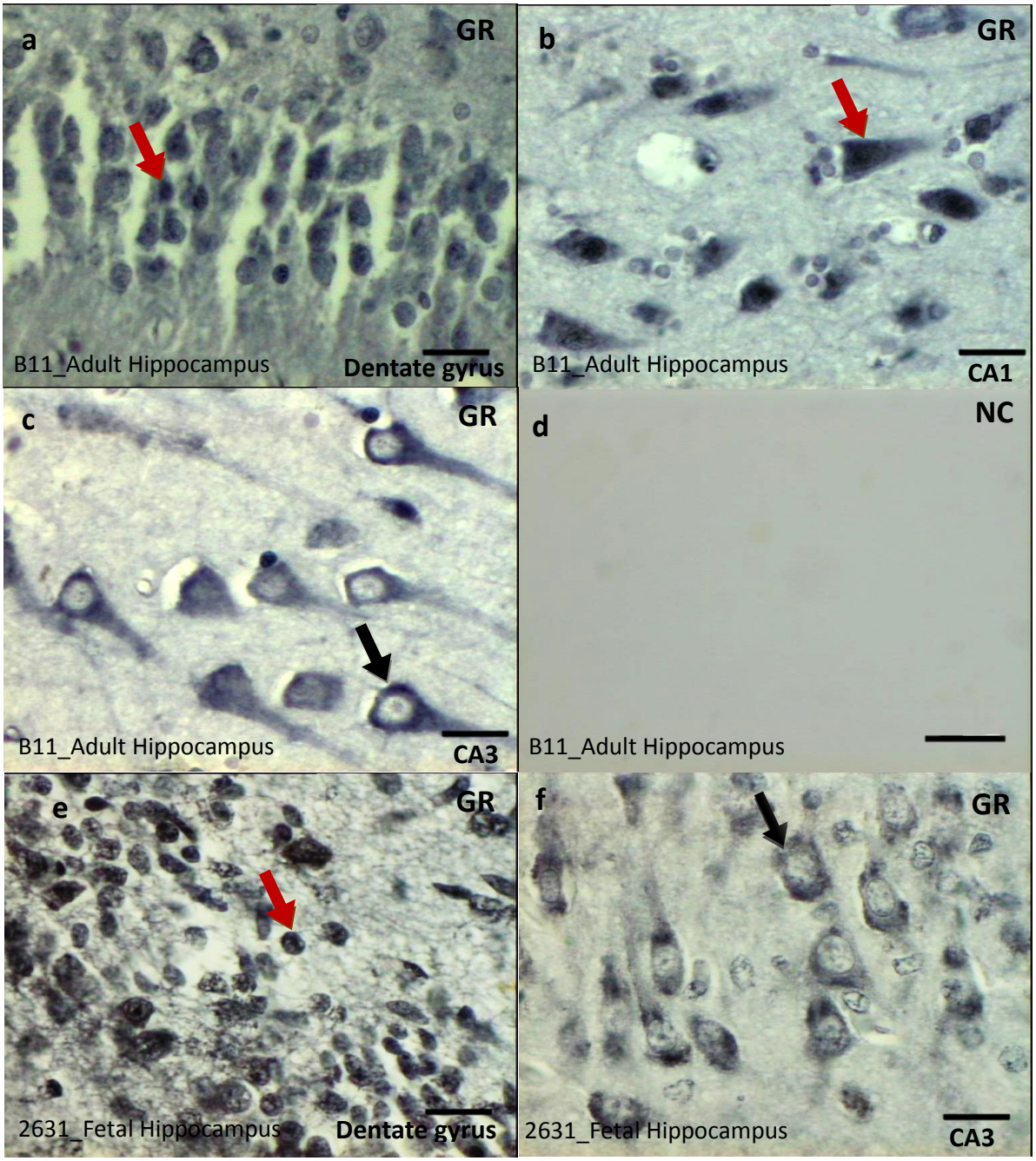

Figure 2. *GR-immunoreactivity in adult and fetal hippocampus.* Coronal sections of adult human hippocampus of case GBB B11 (**a – d**) and of fetal human hippocampus of GBB 2631/09 (38 w, HII group 1) (**e** and **f**). In figures **a**, **b** and **c**, GR-IR neurons are shown at different areas of the adult hippocampus: dentate gyrus (**a**), CA1 (**b**) and CA3 region (**c**) with both nuclear and cytoplasmic GR distribution. Note the intense signal in the nuclei of hippocampal neurons in the dentate gyrus. Figure **d** depicts a negative control section with no reaction found. In figures **e** and **f**, fetal human hippocampus was stained for GR. In the dentate gyrus (**e**), GR labeling was intense in the nucleus, whereas in CA region, GR was mainly detected in the cytoplasm. Note also the punctate nuclear distribution. Red arrows indicate the nuclear distribution of GR in the hippocampal neurons and black arrow the cytoplasmic one. CA = cornu ammonis, GR = glucocorticoid receptor, NC = negative control. Scale bar (a – c, e – f) = 20 μm, scale bar (d) = 50 μm

GR-immunoreactivity in the fetal Locus coeruleus

In the LC of the human neonate all types of cells were positive for GR immunoreactivity including neurons and glial cells. The majority of TH-IR neurons (Fig. 3a) displayed GR staining in both nucleus (red arrows) and cytoplasm (green arrow) (Fig. 3b). GR-staining showed variability among the cases in intensity as well in distribution.

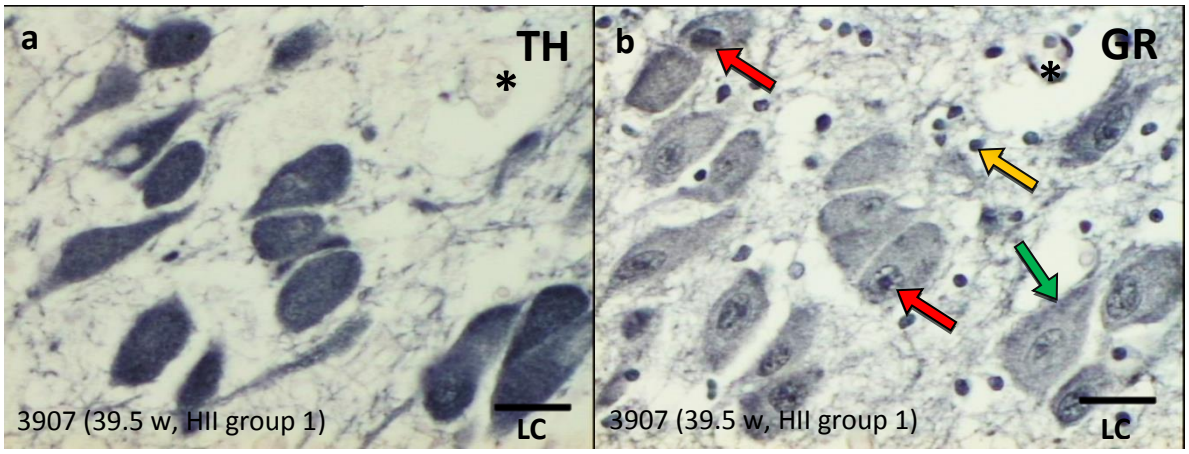

Figure 3. *GR distribution in the TH-IR LC neurons*. Two adjacent sections at the caudal level of the LC of case GBB 3907/07 (39.5 weeks of age, HII group 1). In figure **a** and **b,** noradrenergic neurons were stained with TH (**a**) and GR (**b**), respectively. Note the nuclear (red arrows) and cytoplasmic (green arrow) distribution of GR. Except for neurons, glial cells were also GR-IR (yellow arrow). TH = tyrosine hydroxylase, GR = glucocorticoid receptor, LC = Locus coeruleus. Asterisks show blood vessels in adjacent sections. Scale bar (**a**, **b**) = 20 μm.

In the "control" case GBB 170/16 (case without HII neuropathological lesions), GR was mainly found in the cytoplasm forming aggregates near the inner part of plasma membrane (Fig. 4a1, red arrows). Some signal was also detected inside the nucleus, particularly in the nucleolus (Fig. 4a2). In neonates with ranging hypoxia grading similar distribution was detected with differences in the intensity of staining. In cases of HII group 1, GR labeling was clear in both cytoplasm and nucleus. More of the cases exhibited moderate nuclear signal and intense aggregates in the cytoplasm (Fig. b1). However, in cases GBB 3907/09 and 2735/09 darker nuclei than cytoplasm were observe with less cytoplasmic aggregates. Note the GR presence in the nucleolus (Fig. b2, green arrow). In cases of HII group 2, LC neurons displayed mainly cytoplasmic distribution with weak nuclear signal (Fig. 4c1). The cytoplasmic aggregates appeared unified in some of the cases, e.g. GBB 237/17 and 311/18 (Fig. 4 c2, red arrow). In samples of HII group 3, the nucleus appeared unstained, although the nucleolus showed to be very weakly stained in some cases. (Fig. 4d2, green arrows). GR staining was mainly cytoplasmic forming aggregates (Fig. 4d1, red arrows).

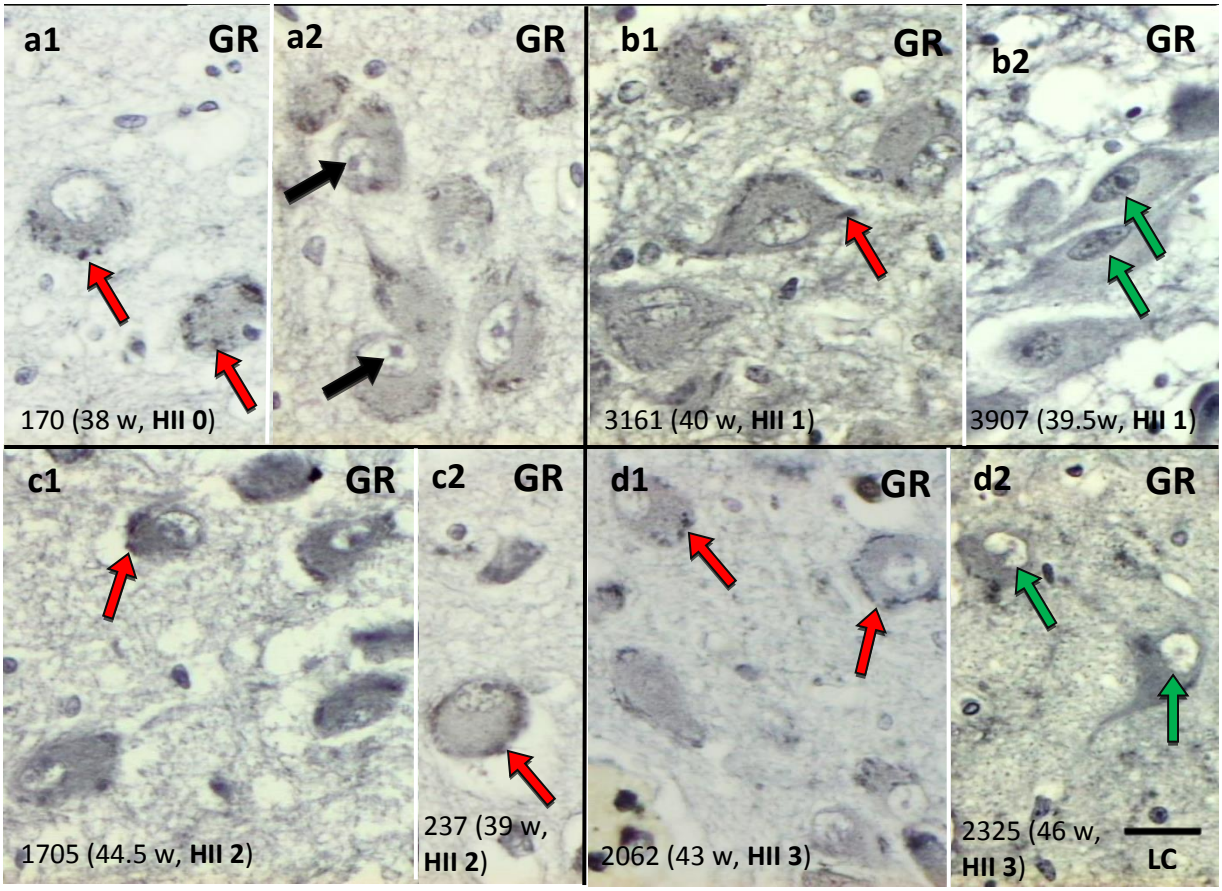

Figure 4. *GR distribution among cases of different HII grading*. In the control case GBB 170/16 (38 w, HII 0, **a1** and **a2**) note the weak signal in the nucleus. GR was mainly found in the cytoplasm forming aggregates (a1, red arrows) and in the nucleolus (a2, black arrows). In HII 1, cases 3161/13 (40 w, HII 1, **b1**) and 3907/07 (39.5 w, HII 1, **b2**), GR was found in both cytoplasm with aggregates (red arrow, **b1**) and in nucleus (**b2**). In HII 2, cases 1705/05 (38 w, HII 2, **c1**) and 237/17 (39 w, HII 2, **c2**), GR was mainly observed in the cytoplasm forming aggregates (red arrows, **c1**). In some cases, aggregates seemed to be unified (red arrows, **c2**). In cases of HII 3, 2062/07 (43 w, HII 3, **d1**) and 2325/07 (46 w, HII 3, **d2**), GR was detected in the cytoplasm forming aggregates (red arrows, **d1**) while nucleus remained unstained in the majority of the cases (green arrows, **d2**). TH = tyrosine hydroxylase, GR = glucocorticoid receptor, LC = Locus coeruleus. HII = hypoxic- ischemic injury. Scale bar = 20 μm.

A clear difference was observed in GR staining intensity between the stillborn and live-born cases. Stillborn neonates exhibited poor signal especially in the nuclear area (Fig. 5b) compared with the live-born ones (Fig.5a).

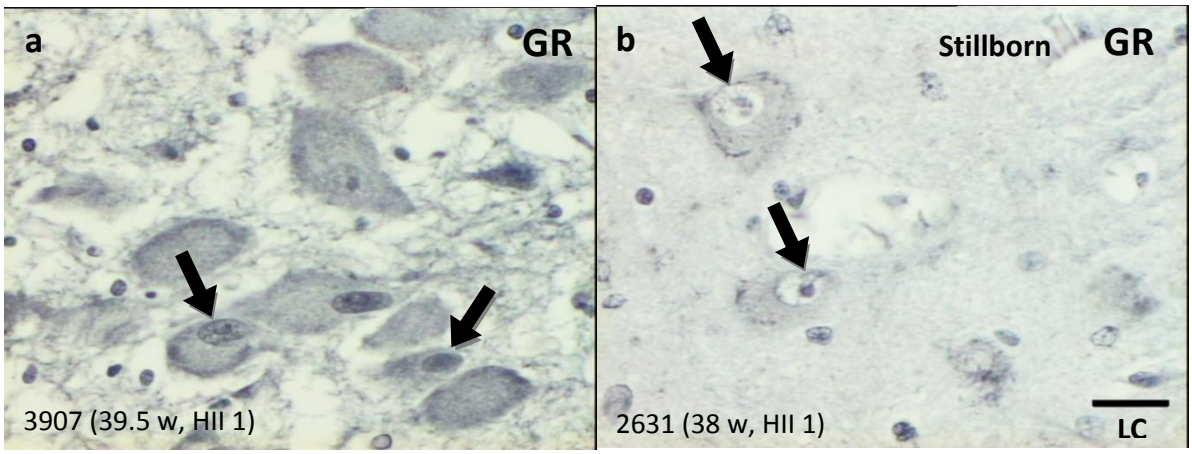

Figure 5. *Comparison of GR labeling between live-born and stillborn cases: Stillborn embryos exhibit poorer GR labeling in the nucleus of LC neurons.* High magnification of LC neurons stained with GR of neonates GBB 3907/07 (39.5 w, HII group 1) live-born, (**a**) and 2631/09 (38 w, HII group 1), stillborn (**b**). Note the reduction of GR staining in the nucleus of LC neurons of the stillborn case compared to the live-born one with the same hypoxia grade. Black arrows show the nuclei of LC neurons. GR = glucocorticoid receptor, LC = Locus coeruleus, HII group = hypoxic- ischemic injury group. Scale bar = 20 μm.

Glucocorticoid receptor was almost found in all types of cells. Noticeable expression of GR was present in ependymal cells around the fourth ventricle with variable intensity between the cases (Fig. 6a and b) as well as in endothelial cells around blood vessels (Fig. 6c).

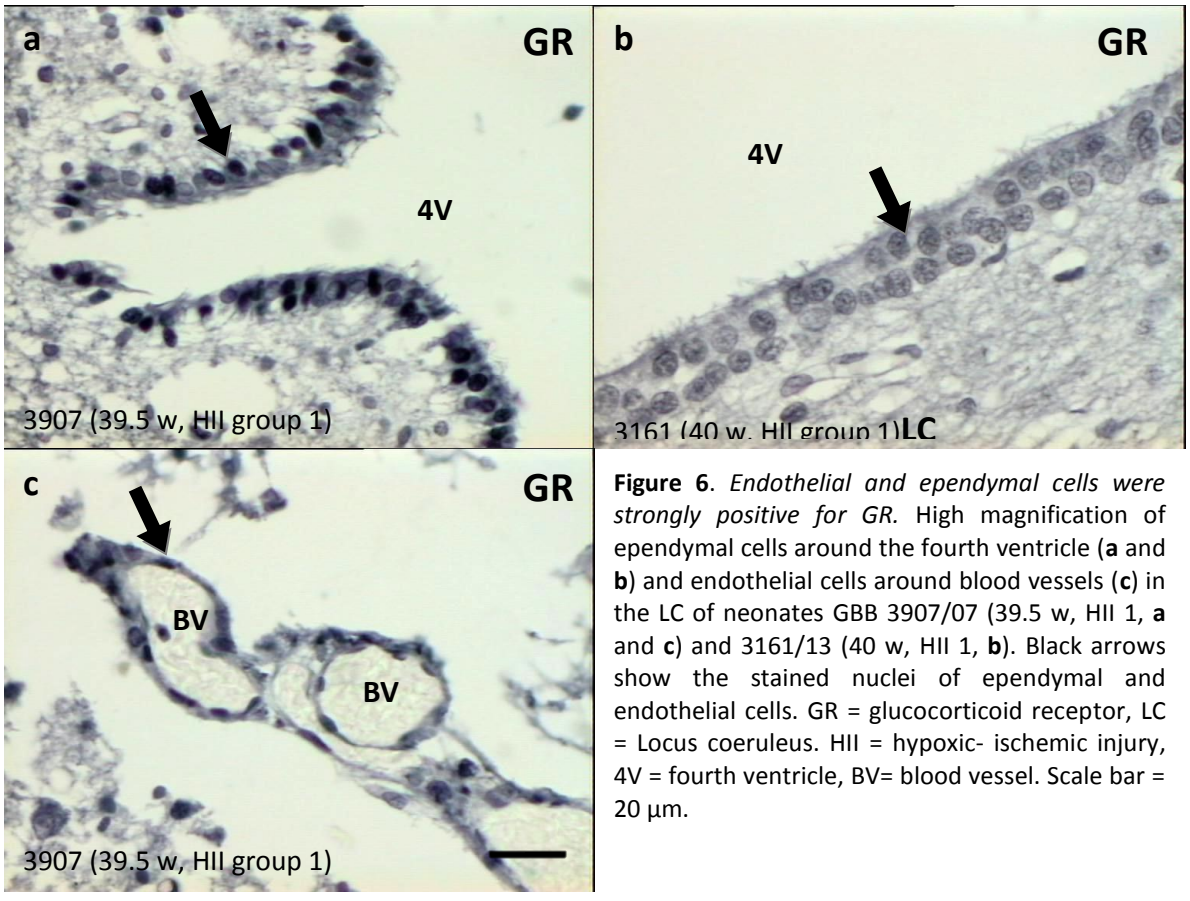

Figure 6. *Endothelial and ependymal cells were strongly positive for GR.* High magnification of ependymal cells around the fourth ventricle (**a** and **b**) and endothelial cells around blood vessels (**c**) in the LC of neonates GBB 3907/07 (39.5 w, HII 1, **a** and **c**) and 3161/13 (40 w, HII 1, **b**). Black arrows show the stained nuclei of ependymal and endothelial cells. GR = glucocorticoid receptor, LC = Locus coeruleus. HII = hypoxic- ischemic injury, 4V = fourth ventricle, BV= blood vessel. Scale bar = 20 μm.

Morphometric and statistical analysis of GR stained sections confirmed some of our qualitative observations and revealed some additional correlations.

Stillborn embryos had low GR immunoreactivity in the nucleus of LC neurons

Statistical analysis revealed a negative correlation between GR OD inside the nuclei of LC neurons and stillbirth (rho= -0.441, p=0.046) (Fig. 7). The signal for GR detection was poor in the nuclei of LC neurons of stillborn cases. On the other hand, in live-born cases the nuclei exhibited intense reaction, as also shown in figure 5a.

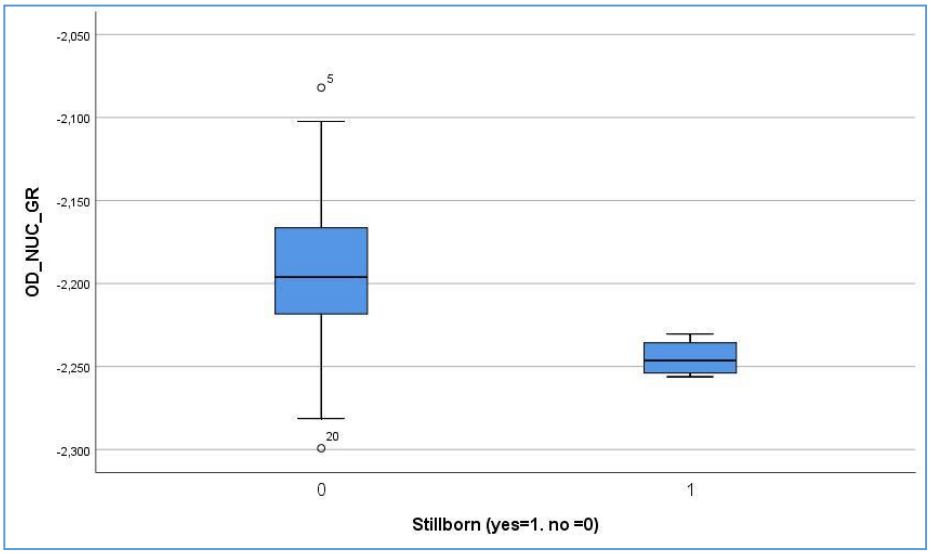

Figure 7. *Stillborn embryos had lower GR immunoreactivity in the nucleus of LC neurons than the live-born ones (rho= -0.441, p=0.046).* Scatter plot displaying the OD for GR measured in the nuclei of LC neurons. The medial lines present the mean OD for each group (non-stillborn and stillborn), which is –2.19 ± 0.01, and –2.24 ± 0.01, respectively. Reduction in the OD for GR was observed in the stillborn infants.

In full-term neonates, GR immunoreactivity in the nucleus of LC neurons exhibited negative correlation with hypoxia grade

By classifying the samples in premature (<37 gestational weeks) and full-term (=>37 gestational weeks), we statistically found that in full-term neonates GR immunoreactivity in the nuclei of LC neurons was negatively correlated with hypoxia grade (rho= -0.576, p=0.025) (Fig. 8). In HII grade 1 cases, GR OD inside the nuclei of LC neurons was high, but as hypoxia was prolonged (HII grade 2 and 3), GR immunoreactivity was weaker.

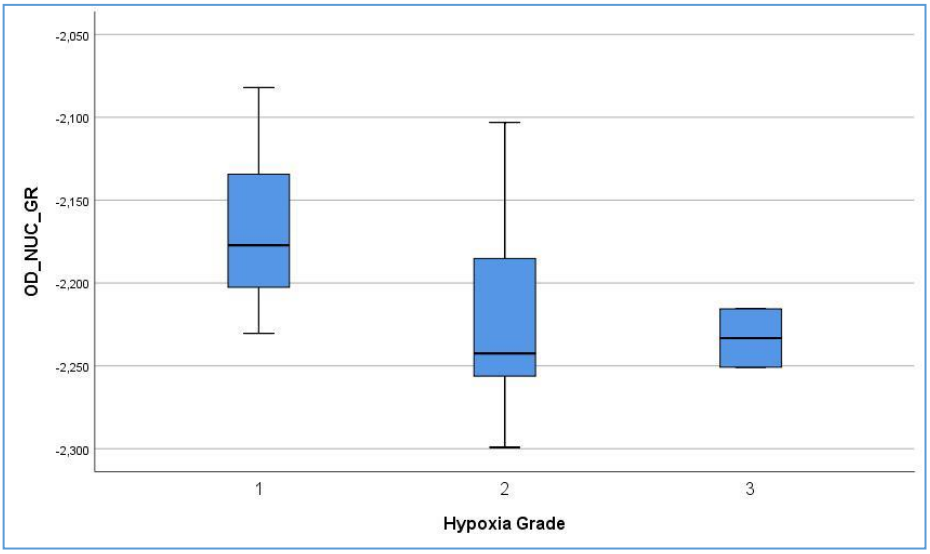

Figure 8. *In full-term infants, GR immunoreactivity in the nucleus of LC neurons exhibited negative correlation with hypoxia grade.* Scatter plot displaying the OD for GR measured in the nuclei of LC neurons. The medial lines present the mean OD for each group, HII grade 1, 2 and 3, which is –2.17 ± 0.02, -2.22 ± 0.03 and –2.23 ± 0.02, respectively. The GR staining was decreased in the nuclei of LC neurons as the hypoxia grade was increased.

Apart from the differences of GR-labeling intensity, statistical correlations regarding the cellular and nuclear size of LC neurons were revealed, as it is presented below.

Cell and nucleus size of LC neurons was negatively affected by hypoxia grade

Morphometric analysis showed that cellular and nuclear size of LC neurons are strongly affected by the hypoxia grade. Specifically, the higher HII grade, the smaller cell (rho= -0.479, p=0.028) and nucleus size (rho= -0.577, p=0.006) (Fig. 9). In HII group 1 (consistent with severe/acute injury), neuronal bodies as well as nuclei appeared smaller compared with HII group 0. In moderate/ more prolonged hypoxia (HII group 2) the measurements seemed to maintain almost the same with group 1. In contrast, in HII group 3 (very severe/long duration or chronic HII), size of both neuronal bodies and nuclei exhibited a great shrinkage compared with all the other hypoxia grade groups.

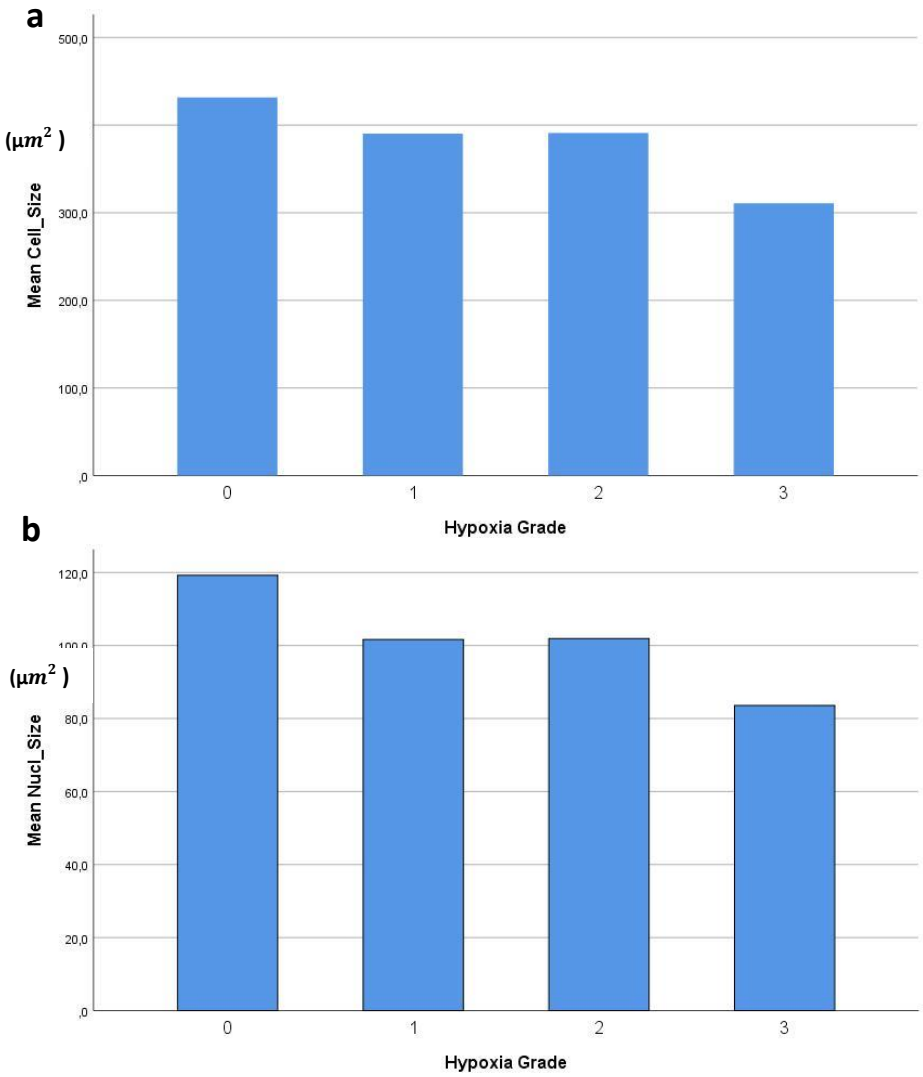

Figure 9. *Cellular and nuclear size of LC neurons was negatively affected by hypoxia grade.* The mean values of cellular (**a**) and nuclear sizes (**b**) in LC neurons for each HII group, as assessed in GR-stained sections. For HII groups 1, 2 and 3, the means ± SEM were 390 ± 25, 391 ± 24 and 311 ± 7, respectively for cellular size and 102 ± 7, 102 ± 6 and 84 ± 2, respectively, for nuclear size. Note that the mean cellular and nuclear sizes are significantly reduced as hypoxia grading is increased.

In premature neonates, cell and nucleus size of LC neurons was decreased in cases with infection and higher hypoxia grade

In premature neonates (<37 gestational weeks), hypoxia grade cellular and nuclear size of LC neurons was negatively correlated with hypoxia grade as it was presented for ensemble the cases (rho=-0.926, p=0.008 and rho=-0.926, p=0.008, respectively). Moreover, infection by viruses or microorganisms appeared to also affect negatively cellular (rho=-0.878, p=0.021) and nuclear (rho= -0.878, p=0.021) size of LC neurons (Fig. 10). That finding was not observed in full-term neonates.

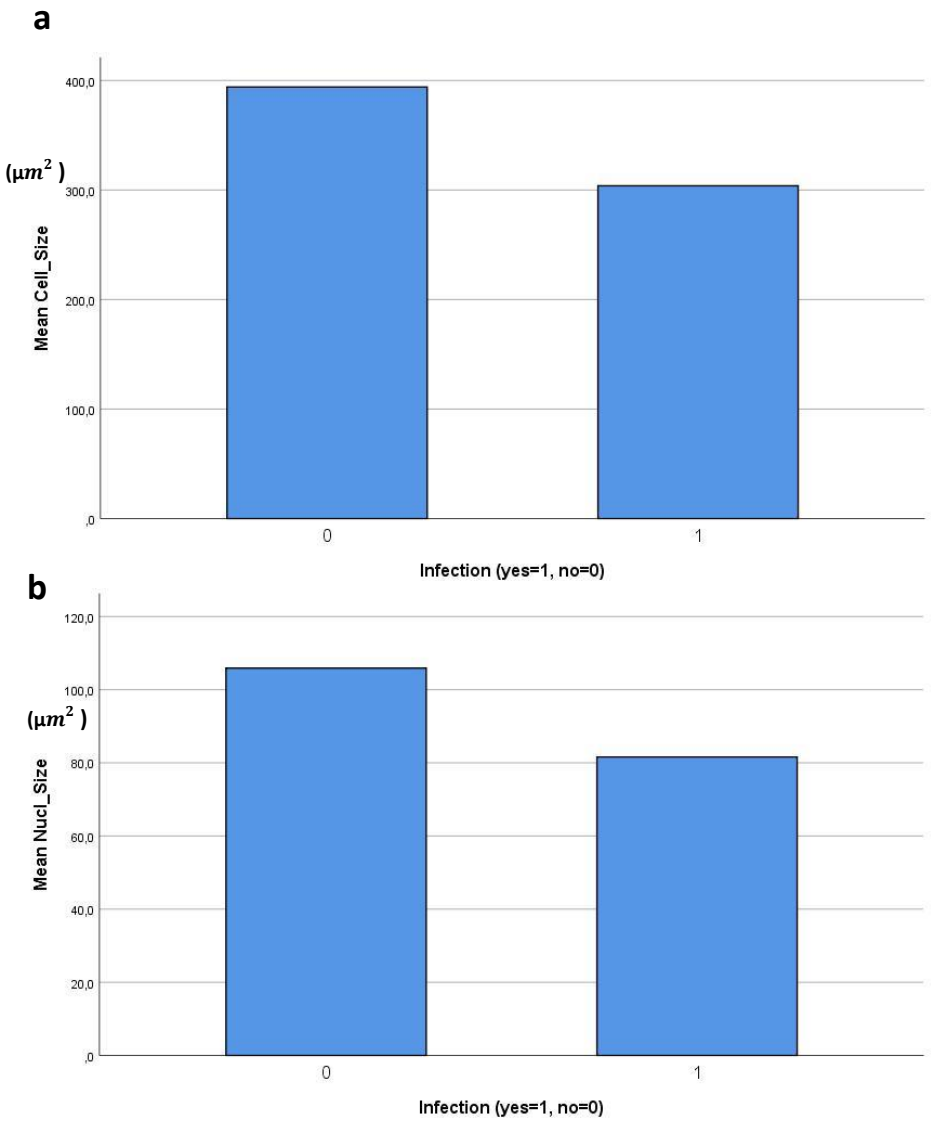

Figure 10. *In premature neonates, cellular and nuclear size of LC neurons was decreased in cases with infection.* The mean values of cell (**a**) and nucleus sizes (**b**) in LC for each group (0= no infection, 1= infection), as assessed in GR-stained sections. For groups 0 and 1 the means ± SEM were 394 ± 20, 304 ± 13, respectively for cell size and 106 ± 7 and 82 ± 4, respectively, for nucleus size. Cases with infection had reduced cellular and nuclear size.

Discussion

Our study showed both qualitative and quantitative changes of GR expression and distribution in the LC of the human neonates.

The application of the anti-peroxidase immunohistochemistry using the antibody ED5 against GR protein in both adult and fetal human hippocampus confirmed the efficiency of the product, in agreement with the Wang et al. (Wang et al., 2013). Hippocampal neurons were clearly positive for GR. In our sample, however, hippocampal neurons were stained not only in the nucleus as shown in Wang et al., 2013, but also in the cytoplasm. That difference is not related to developmental processes, as it is observed in both adult and fetal human samples. Although we followed the same procedure, we used a different GR antibody from the same company (monoclonal, ED5), because antibody used by Wang et al., (polyclonal, E-20) is not anymore commercially available. This change may play a role in the observed additional cytoplasmic distribution, even though both antibodies were designed against peptides in the N-terminus of GR.

Except for the neurons, also other kind of cells showed intense GR labeling, such as glial cells. Ependymal cells of fourth ventricle and endothelial cells of the blood vessels were strongly positive. Experiments in rats showed that GR expression in ependymal cells seems to be involved in glucose transport and glucose sensing system via a ligand dependent way (Iwata and Ozawa, 2014). Furthermore, it is known that corticosteroids act via GR to improve the barrier properties of endothelial cells upregulating the expression of tight junction proteins, occludin and claudin-5 and adherens junction protein VE-cadherin (Salvador et al., 2014).

Intense GR labeling was observed in the noradrenergic neurons of the human neonates. In the cytoplasm, the staining was not homogeneous, but was found forming structures - like GR aggregates – located mainly in the inner part of plasma membrane. Similar aggregates were observed in HeLa and COS-7 cell lines where GR aggregation and degradation in cytoplasm had been promoted by GR Hsp90 inhibition (Prima et al., 2000). Molecular studies indicate that inactive GR in the cytoplasm interacts with several proteins, as chaperones (hsp90, hsp70), co-chaperones (hip, hop), immunophilins (FKBP59, Cyp40) and others (p23, tubulin) (Prima et al., 2000), (Akner et al., 1995). Main neuropathological alterations after hypoxic brain injury are known to include protein misfolding and aggregation due to the sharp reduction of ATP levels, which are required for the action of chaperons leading to termination or downregulation of co-translational folding, ubiquitin-proteasome-mediated degradation and autophagy (Hua et al., 2017). Thus, the blocking of proper protein folding is possibly linked to the underling mechanism of aggregates' formation.

In the nucleus of LC neurons of human neonates, we observed that activated GR was mainly found in the nucleoli and in smaller clusters. In experimental models, GR forming clusters was observed in the nucleoplasm (Steensel et al., 1995) dynamically interacting with nuclear bodies enriched in the co-regulator NCoA-2, DNA-dependent foci and chromatin targets (Stortz et al., 2017).

Reduced GR staining was observed in the stillborn compared to the live-born neonates, which reaches statistical significance only for the nucleus. That could be explained by the autolysis: as embryos died inside the uterine and stayed there until their birth in constant temperature 37 °C, it is expected that degradation of proteins has started and production of new ones has been blocked. Consequently, the number of GRs is low and the signal is poor.

In full-term neonates, OD of GR in the nucleus of LC neurons exhibited negative correlation with hypoxia grading. In acute hypoxia, GR immunoreactivity in the nucleus was higher than in moderate or prolonged hypoxia. Considering that when ligand (cortisol) is absent, GR is mainly found in the cytoplasm and translocation in the nucleus occurs when it binds it, we suggest that noradrenergic neurons of the human neonates are activated via cortisol in HII group 1 cases. GR-mediated glucocorticoid action takes place in phase 3 and 4 of stress response, which occurs within minutes or hours after the stressful stimulus (de Kloet and Meijer, 2019). As newborns died during the acute phase of hypoxia, GR upregulation is reasonable in HII group 1. However, in prolonged hypoxia (HII group 2 and 3) GR immunoreactivity is low. GR may have been down-regulated in a compensatory way due to the excessive cortisol levels of the initial phase (Hannibal and Bishop, 2014) or medical treatments received in the intensive care hospital units.

It is interesting that both GR and TH (Pagida et al., 2016) are found to be up-regulated in acute hypoxia phase. In the early phase of stress response, norepinephrine is released from LC (Hannibal and Bishop, 2014). TH expression has been connected with cortisol action since several studies indicated that noradrenergic neurons in the LC may be target cells for glucocorticoids, and that a GR-mediated mechanism may underline the glucocorticoid effect on TH (Markey et al., 1982), (Hensleigh and Pritchard, 2013), (Busceti et al., 2019).

The morphometric and statistical analysis showed that cellular and nuclear size of LC neurons was decreased as perinatal hypoxia gets prolonged supporting the results of previous studies conducted by our laboratory (Pagida et al., 2016). The size reduction seems to be independent of postmortem delay or fixation time as also reported in the hypothalamus (Ganou et al., 2010), substantia nigra and LC (Pagida et al., 2013, 2016). Chronic hypoxia as an adaptive mechanism triggers changes in metabolism resulting in the observed reduction in cell size (Hochachka et al., 1994), (Schwartz et al., 2004).

Apart from perinatal hypoxia, infection was found to affect cellular and nuclear size in premature neonates. Preterm newborns are at high risk of infection (Collinsa et al., 2018) since they have an immature antioxidant system, as long as the maturation and upregulation of the antioxidant system takes place during the late pregnancy (Martin et al., 2018). Recent studies pointed out that both infection and perinatal hypoxia could cause brain damage via common cellular and molecular pathways, involving the production of reactive oxygen species (ROS). Infection may lower the threshold at which hypoxia alone triggers brain injury (Coimbra-costa et al., 2017), (Novak et al., 2018), (Ugwumadu, 2006), (Zhao et al., 2013), (Kendall and Peebles, 2005).

In summary, regardless of heterogeneity of our samples concerning the gestational/postnatal age, the congenital conditions and medications experienced by the human fetus through both gestation and delivery, our study demonstrates that in full-term neonates, GR immumoreactivity in the nucleus of noradrenergic neurons is negatively correlated with hypoxia grading. In the acute hypoxic phase, GR is upregulated and translocated in the nucleus. Moreover, stillbirth causes decrease of GR immunoreactivity in the nucleus of LC neurons probably due to GR-related protein degradation inside the uterus. Since LC is a brain area directly participating in stress response (Benarroch, 2009) and GR is a crucial molecule mediating that reaction (de Kloet and Meijer, 2019), our study contributes to understanding the pathways of stress response in infants experienced perinatal hypoxia. Given that epigenetic modulations of GR protein during the critical perinatal period may be involved in the onset of neuropsychiatric and/or neurological disorders later in life (Lewis and Olive, 2014), (McGowan et al., 2009), (Li et al., 2012), (Turecki and Meaney, 2016), (Vaiserman and Koliada, 2017), our findings may have important clinical impact on the etiopathology of stress-related disorders. Additional experiments regarding other brain areas directly implicated in stress response, as hypothalamus and hippocampus, and other proteins acting in combination with GR and affecting cortisol function, such as mineralocorticoid receptor (MR) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2) (Seckl, 2001), must be done, in order to compose the different pieces of that complicated puzzle.

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