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Stress-in-a-dish

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STRESS-IN-A-DISH

MODELING THE NEUROBIOLOGY OF GLUCOCORTICOIDS *IN VITRO*, INVESTIGATING STRESS SUSCEPTIBILITY, AND HIGHLIGHTING ETHICAL IMPLICATIONS

KATHERINE BASSIL

STRESS-IN-A-DISH

Modeling the neurobiology of glucocorticoids *in vitro*, investigating stress susceptibility, and highlighting ethical implications

Katherine Bassil

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STRESS-IN-A-DISH:

Modeling the neurobiology of glucocorticoids *in vitro*, investigating stress susceptibility, and highlighting ethical implications.

DISSERTATION

To obtain the degree of Doctor at Maastricht University,
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Chapter 1

General Introduction

Stress-related disorders

Trauma and stress-related disorders (abbreviated as SRDs), including post-traumatic stress disorder (PTSD), have become a major public health concern, affecting a large proportion of the population worldwide[1], especially the youth[2]. The prevalence of SRDs has increased considerably, with an estimation of a 25% increase, following the recent COVID-19 pandemic[3]. PTSD prevalence, in particular, was estimated at 6.8% pre-pandemic[4], and is now estimated at 17.52% post-pandemic among COVID-19 patients, health professionals, and the population at large[5]. Based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), PTSD develops following exposure to a traumatic event including war, sexual abuse, childhood abuse, (natural) accidents, among others[6]. Symptoms of PTSD include intrusive thoughts, hyperactivity, avoidance, and negative alterations in mood[6], and often overlap with symptoms of other SRDs, namely major depression and anxiety disorders[7, 8].

> *"It was a little bit of a shell shock. Everything just happened so fast. It just didn't give us time to cope with everything that was going on," said the mother of two. Life and death decisions of who would get a ventilator were made in seconds and multiple times a day. "It literally felt like we were in war."[9]*

Not all individuals exposed to a traumatic event or prolonged exposure to stress develop SRDs, particularly PTSD: a small proportion of susceptible individuals will develop neurobiological and behavioral deficits, while the majority will be able to adapt and show resilience[10]. Although the concepts of susceptible and resilient phenotypes are well known, the molecular mechanisms underlying differential vulnerability to develop PTSD have not been resolved yet. Several risk factors are suggested to influence the risk of developing PTSD[11]. Pre-trauma risk factors - risk factors that are preexisting prior to experiencing a trauma - include underlying (epi)genetic vulnerabilities, existing mental health or neurological conditions, previous exposure to adversity including childhood trauma, and lower intelligence or educational attainment[12, 13]. Gene-environment interactions are crucial in determining the likelihood of developing SRDs. That is, genetic liability increases an individual's susceptibility to SRDs when confronted with the above mentioned adversities[14]. Particularly, epigenetic mechanisms have been suggested as playing a mediating role between trauma exposure and stress susceptibility[15].

For instance, studies looking into underlying biomarkers for PTSD susceptibility and resilience are on the rise, with the hope to identify biomarkers that could serve as predictors for risk to PTSD. Different types of biomarkers are being investigated for PTSD, including molecular and cellular biomarkers for instance neurogenesis in the prefrontal cortex (PFC);

genetic markers including genetic polymorphisms in the *FKBP5* gene, in addition to epigenetic markers such as differential DNA methylation in candidate genes such as *DUSP22* and *ZFP57*[16]. These efforts serve as ways to provide early interventions for preventing the occurrence of PTSD, as opposed to alleviating or treating existing PTSD symptoms[17].

A key risk factor for developing SRDs is the exposure to early life stress (ELS) such as child abuse and neglect[18, 19]. Vulnerabilities to psychiatric disorders in general, emanate from gene-environment interactions, particularly during critical periods of brain development, where neuroplasticity mechanisms are influenced by environmental challenges such as stress[20]. The human brain goes through dynamic changes during development and exposure to stress, particularly repetitive and chronic stress, during this critical period can lead to increased vulnerability for psychiatric disorders in later life[21].

> *"Many abused children cling to the hope that growing up will bring escape and freedom. But the personality formed in the environment of coercive control is not well adapted to adult life. [...] She is still a prisoner of her childhood; attempting to create a new life, she reencounters the trauma." — Judith Lewis Herman*

Cognitive networks such as that formed between the PFC and the structures of the limbic system - involved in the regulation of emotion and social behavior - seem to be vulnerable to the effects of stress[22, 23]. For instance, chronic ELS has been associated with negative alterations in dendrite morphology in rodents[24], and a reduced volume and connectivity of the PFC in humans[25], further increasing the risk of human adolescents and young adults to future SRDs. Interestingly, the PFC is one of the brain regions where stress-induced changes are considered to be more reversible than changes in the amygdala[23], which makes it an important region to investigate in light of chronic stress effects. The reversibility of stress-induced effects in the PFC are mainly due to the interconnection between the limbic system and the hypothalamic pituitary adrenal (HPA) axis[26], a key player in the stress response. The underlying mechanisms of stress susceptibility to PTSD and SRDs are complex and not fully understood, but it is believed that disruptions in the normal functioning of the stress response play a critical role.

The stress response

During a stressful experience, the hypothalamus stimulates the autonomic nervous system responsible for the "fight or flight" response, in addition to the HPA axis which is responsible for the regulation of cortisol (CORT) levels[27]. Once the HPA axis is activated,

the hypothalamus triggers the release of corticotrophin-releasing hormone (CRH) which stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) which is released in the blood stream. Finally ACTH binds to the adrenal cortex inducing the release of glucocorticoids (GCs) such as CORT in humans[28]. GCs act on various target tissues, including the brain, to modulate the stress response and maintain homeostasis. Under basal conditions, the HPA axis is involved in functions such as growth, immunity, and development. Under stressful conditions, heightened HPA-axis activity leads to enhanced release of CORT that mobilize resources and energy while suppressing functions that are not required for immediate survival. The level of CORT is regulated via negative feedback at several brain regions of the HPA axis. Together, the autonomic nervous system and the HPA axis, work to regulate the stress response. Interestingly, a dysfunctional HPA axis, either decreased activation or increased activation, has been associated with the development of psychiatric disorders. For instance, depression and PTSD are characterized by hyposuppression and hypersuppression of the HPA axis, respectively[29].

GCs act on two types of receptors: the low affinity glucocorticoid receptor (GR) and the high affinity mineralocorticoid receptor (MR). Together their activation brings about both genomic and non-genomic changes in cells[30]. The genomic effects of GCs involve the regulation of gene transcription resulting in long-lasting changes in gene expression, which further alters the function of cells. Non-genomic effects are rapid and include changes in intracellular signaling pathways, and the modulation of the activity of enzymes and transporters[31]. GR and MR control a number of neuronal processes including proliferation, neuronal differentiation, and excitability, eventually impacting mood, cognition and behavior functions, all crucial in the regulation of acute or chronic stress[32]. Additionally, the GR plays a big role in negative feedback regulation of CORT release[33].

Given the high prevalence of SRDs, namely PTSD, and the growing evidence of the role of ELS as a risk factor[34], understanding the underlying mechanisms of stress during neurodevelopment is critical. The developing brain is particularly susceptible to the effects of GCs and there is growing evidence that early life exposure to high levels of GCs can have long-lasting effects on brain structure and function[35].

Role of GCs during neurodevelopment

At baseline levels, GCs are essential for the regulation of several neuronal processes in the developing brain, including neural progenitor cell proliferation, differentiation, and

maturation. They mediate the formation of new neuronal connections, synaptic plasticity, and overall brain maturation[36]. For instance, GCs can cause changes to neuronal plasticity mechanisms in several brain regions, including the PFC[37, 38]. Moreover, GCs have been shown to promote the expression of brain-derived neurotrophic factor (BDNF) [39, 40], which is important for promoting survival and differentiation of neurons[41], and genes involved in cell cycle regulation[42], in addition to the inhibition of apoptosis-related genes[43]. Studies have even shown that circadian fluctuations in GC levels have a positive effect on synapse turnover in the cerebral cortex, thereby influencing learning systems. Additionally, GCs act within the stress response system, to provide a survival advantage for the developing brain, by promoting adaptive responses to stressors, and providing resilience to future stressors: a phenomenon known as allostasis[44].

However, excessive, or prolonged exposure to GCs can lead to negative consequences, including disruptions in neurodevelopmental processes. This maladaptation is typically referred to as allostatic overload. For example, several *in vivo* and *in vitro* studies have illustrated the negative effects of excess GCs on neural cell proliferation, neurogenesis, and synaptic transmission[42]. The neurodevelopmental effects of GCs partly match with the neurodevelopmental effects of stress as a psychological experience, suggesting the importance of looking into the effects of GCs during neurodevelopment as a mediator for SRDs[45]. Early stages of neuronal development are highly sensitive to the effects of GCs which can be seen through changes in brain regions of the limbic system, including the amygdala, and hippocampus. Additionally, GCs impact synaptic transmission with chronic stress negatively regulating glutamatergic synaptic transmission. Research indicates that increased exposure to GCs during early development can result in cortical thinning, reduced cortical folding, and pronounced depressive symptoms later in life. Furthermore, the effects of GCs on neurodevelopmental processes are similar to those of psychological stressors, suggesting that GCs play a crucial role in pathological mechanisms of SRDs in the case of ELS[45]. Therefore, studying the role of GCs during neurodevelopment could help identify underlying susceptibility mechanisms to SRDs.

Advanced *in vitro* models of neurodevelopment: human pluripotent stem cells

The use of stem cells for in-vitro modelling of brain processes has regained attention since the Nobel-prize winning achievement of induced pluripotent stem cells (iPSCs) in 2006. Shinya Yamanaka was one of the first to demonstrate that adult somatic cells can be reprogrammed to iPSCs through viral transduction of only four transcription factors (i.e., *OCT4*, *SOX2*, *KLF4*, and *cMYC*)[46]. PSCs (including human embryonic stem cells (hESCs) and iPSCs), are characterized by self-renewal and an ability to differentiate into the three

germ layers: endoderm, mesoderm, and ectoderm. Following the discovery of this stem cell technology, protocols for the differentiation of PSCs into neuronal populations were rapidly established. The reprogramming of adult somatic cells, including fibroblasts and blood cells, into iPSCs have opened avenues of the generation of disease-specific central nervous system (CNS) *in vitro* models for relevant neurological and psychiatric diseases. In fact, PSC-derived models offer advantages beyond what previous *in vitro* models were able to achieve, especially in the modeling of complex psychiatric disorders such as SRDs. For instance, these models allow the generation of improved neuronal cultures characterized by phenotypes that highly resemble *in vivo* conditions. In addition, the variety of neuronal and non-neuronal CNS cells that can be generated from PSCs makes it a promising model for the investigation of several neurobiological mechanisms associated with SRDs, including the impact of GCs on neurodevelopmental processes.

The generation of *in vitro* human stem cell-based models has created novel and advanced opportunities for the study of neurodevelopmental processes and disorders through a method known as indirect conversion. With this method, the differentiation of human PSCs into neurons, goes through separate and defined stages of NPC populations that highly resemble the stages of neurogenesis *in vivo* (**Figure 1**)[47]. That being said, these cellular models allow the study of distinct stages of neuronal development in the context of disease hallmarks, and/or the investigation of the effects of drugs and compounds (e.g., GC) on distinct neuronal processes. Additionally, the advanced method of generating three-dimensional (3D) models namely cerebral organoids, allows a more thorough look into the more complex nature of brain development, including a heterogeneity of cells and neuronal subtypes, and with cerebral organoids being characterized by an increasingly complex cytoarchitecture that highly resembles *in vivo* conditions, as compared to its 2D counterpart[48].

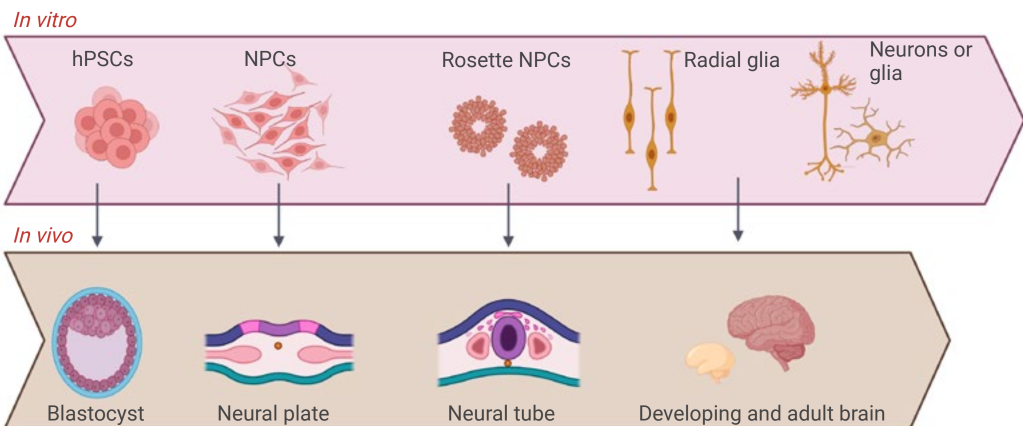

Figure 1. Comparison between stages of neural differentiation *in vitro* and *in vivo*. Adapted from Mertens et al.[47]. (Figure created with BioRender.com)

Beyond the advantages of stem cell technology to generate a human *in vitro* neurodevelopmental model, this technology also holds promise for overcoming scientific and translational challenges in relation to SRDs. To date, the majority of SRD-related studies have relied on either *in vivo* rodent stress models, or on *in vitro* cell lines, namely primary neurons, immortalized human cell lines, and post-mortem brain tissue. Although animal stress models have shown certain neuropathologies and the manifestation of SRD-like symptoms, they have not been useful or successful in identifying novel treatments for patients with SRDs[49]. Moreover, *in vitro* cell lines including immortalized cell lines and post-mortem tissue, offer the advantage of investigating stress-related mechanisms in human cells. Nevertheless, these cell lines carry several limitations and do not express phenotypes that accurately reflect *in vivo* conditions. On the other hand, PSCs (and particular iPSCs), offer avenues for investigating patient- and disease-specific mechanisms involved in SRDs, with the advanced possibility of direct manipulation to better map cause-effect relationships[50].

Ethical, legal, and societal implications (ELSI) associated with *in vitro* modeling of SRDs

Ethics of PSC-derived models

The inception and development of human iPSCs, mitigated the need for using, and thereby sacrificing, human embryos. This technology was considered by many as the solution to the intense, seemingly insolvable debate on the use of embryos to extract PSCs for research purposes. Interest in iPSC models and their increasing research potential for the study of neuronal development in health and disease states, *in vitro* and in animal models (i.e., chimeras), is on the rise with 3D cerebral organoids at the forefront. Characterized by self-organizing abilities, improved potential to model human neurodevelopment, and successful generation of human-rodent cerebral organoid chimeras. With these advances in the stem cell field, the same ethical questions that this technology once promised to evade, are resurrected, and is sparking debate[51]. The use of these systems in modeling human neurodevelopment in the context of SRDs, raises unique ethical questions due to the nature of stress research and the potential to inflict harm, as human and animal research ethics have demonstrated in the past. Highlighting these ethical considerations might bring about a better understanding of best practices in the use of these models in the context of stress research.

Ethics of biomarkers for PTSD

Beyond research-related ethical issues, stem cell models facilitate the identification of biomarkers by comparing iPSC-derived 2D or 3D cells from healthy and SRD patients, or through direct manipulation of these cells to better understand the role and function

of potential biomarkers in human neurodevelopment. Eventually, the identification of biomarkers for SRD susceptibility, might raise ethical questions about the potential (mis) use of this technology in particular contexts. For instance, biomarkers for PTSD susceptibility in the military or law enforcement could increase stigma, discrimination, and even lead to social and professional exclusion[52]. Thinking through the ethical, legal, and social implications (ELSI) of introducing biomarker testing for susceptibility before the translation of this technology to the clinic, could aid in implementing responsible policies and clinical practices.

> *"I honestly don't know how I'm going to feel…if I'm told I have a gene for Alzheimer's…I have no idea how I'll feel about it until it happens. So, I may not want to just like say, 'okay, everybody. Bye, I'm going back to work now'…I just think I didn't realize how I would want it until right now." — Parent interviewed about genetic testing [53]*

Aims and research questions of the thesis

This thesis focuses on filling the gap of knowledge in the available literature investigating the neurobiological effects of GCs *in vitro* in relation to SRD development after ELS and potential ethical implications that may result from this research (for an overview, see **Figure 2**). Specifically, this thesis aims to:

1. Develop an *in vitro* model of ELS to (i) investigate the neurobiological effects of chronic cortisol exposure in human cortical neurons at different stages of neurodevelopment and (ii) to study candidate genes associated with PTSD susceptibility.
2. Highlight the ethical implications associated with the use of advanced human stem cell models for stress-related research and the identification of biomarkers for PTSD susceptibility.

In order to address these aims, the following research questions were raised and addressed:

1. What are the neurobiological mechanisms following GC exposure that are investigated *in vitro*?
2. What are the neurobiological effects of chronic cortisol in human cortical neurons throughout neuronal development?
3. Could an *in vitro* model of ELS be used for investigating candidate genes involved in stress susceptibility?
4. What are the challenges and future considerations for improving the use of *in vitro* models for better understanding GC-related mechanisms implicated in SRDs?
5. What are possible ethical implications in the use of advanced human stem cell models for stress-related research and identification of biomarkers for PTSD susceptibility?

Outline of the thesis

To address RQ1, the review in **Chapter 2** provides an overview of current *in vitro* models used for the investigation of the neurobiological effects of GCs. This review highlights the great variation between studies and lack of replication of key findings, while highlighting advantages and limitations between different *in vitro* models as guidance for future studies.

In **Chapter 3**, RQ2 is addressed by investigating the chronic effects of CORT on neurobiological mechanisms of hESC-derived cortical neurons at different stages of neuronal development, including NPCs, immature differentiating neurons, and maturing neurons. Several molecular and cellular assays were performed to assess stage-specific effects of chronic CORT on neuronal processes including proliferation, apoptosis and cell survival, neurogenesis, synaptogenesis, neuronal activity, in addition to genome-wide transcriptional modifications. This chapter discusses the stage-dependent effects of chronic CORT on key neurobiological mechanisms crucial during neuronal development.

Chapter 4 further addresses RQ2 and explores the molecular trajectories driving neuronal differentiation. This chapter aims to better understand the interaction between neuronal differentiation and the effects chronic CORT, using the established ELS *in vitro* model (as described in Chapter 3). Of the genes driving neuronal development, we identify chronic CORT-sensitive genes in early and late differentiation stages, and we explore the relationship between known genetic variances underlying psychiatric disorders associated with stress as a risk factor.

The exploratory study in **Chapter 5**, addresses RQ3 and aims to apply the ELS *in vitro* model described in Chapter 3 as a model for investigating the effects of CORT on candidate genes associated with PTSD susceptibility, including *ZFP57*, and *DUSP22*. The findings of that chapter suggest the potential of using this ELS model for future *in vitro* studies investigating SRD mechanisms and biomarkers.

An overview of current challenges, limitations, and future perspectives on the use of *in vitro* models for investigating the neurobiological effects of GCs is described in **Chapter 6**, in response to RQ4, including some recommendations on how best to reduce variation and increase reproducibility between studies.

Chapter 7 provides insights into ethical considerations regarding the use of advanced stem cell models, namely 3D cerebral organoids for the investigation of stress-related mechanisms and disorders. The focus of this chapter lies on ethical implications of

cerebral organoids in the context of stress research including research ethics considerations, donor-related issues, and the generation of animal chimeras. This chapter partially answers RQ5.

For a proactive approach into the identification of biomarkers for PTSD susceptibility, **Chapter 8** provides a look into potential ethical implications of identifying PTSD susceptible and resilient individuals (facilitated through biomarker research) for employment purposes, particular in the context of the military and law enforcement agencies. RQ5 is also addressed in this chapter on the neuroethics of biomarkers.

Finally, in **Chapter 9**, I discuss the key findings of this thesis, the overall strengths and limitations, and future perspectives for this line of research.

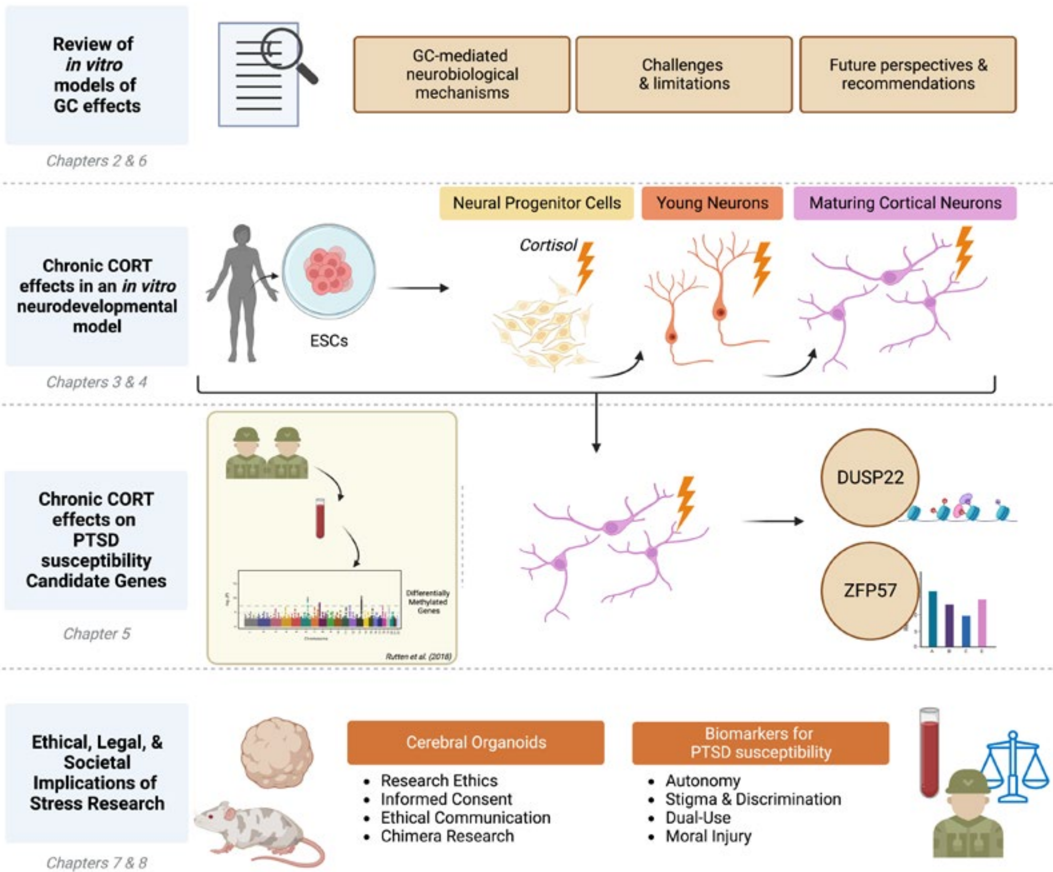

Figure 2. Overview of the studies presented in this thesis. (Figure created with BioRender.com)

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Part I

In vitro Stress Modeling

Chapter 2

In vitro Modeling of the Neurobiological Effects of Glucocorticoids*:* A Review

Based on publication:

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Abstract

Hypothalamic-pituitary adrenal (HPA) axis dysregulation has long been implicated in stress-related disorders such as major depression and post-traumatic stress disorder. Glucocorticoids (GCs) are released from the adrenal glands as a result of HPA-axis activation. The release of GCs is implicated with several neurobiological changes that are associated with negative consequences of chronic stress and the onset and course of psychiatric disorders. Investigating the underlying neurobiological effects of GCs may help to better understand the pathophysiology of stress-related psychiatric disorders. GCs impact a plethora of neuronal processes at the genetic, epigenetic, cellular, and molecular levels. Given the scarcity and difficulty in accessing human brain samples, 2D and 3D *in vitro* neuronal cultures are becoming increasingly useful in studying GC effects. In this review, we provide an overview of *in vitro* studies investigating the effects of GCs on key neuronal processes such as proliferation and survival of progenitor cells, neurogenesis, synaptic plasticity, neuronal activity, inflammation, genetic vulnerability, and epigenetic alterations. Finally, we discuss the challenges in the field and offer suggestions for improving the use of *in vitro* models to investigate GC effects.

Keywords: stress, stress disorders, glucocorticoids, in vitro models, psychiatry, neurobiology

Introduction

Stress and stress-related disorders

Stress can be defined as any change to the environment, either internal or external, that may lead to homeostatic disruption or imbalance. This definition takes into account variations that may accompany individual stress responses and disparate effects of a single stress stimulus[1]. The relationship between stress and ill-health is not straight-forward. Stressors can elicit various responses depending on a number of factors that include, but are not limited to sex, developmental time-window of the exposure, genetics and type and length of the stressor. For example, acute stress has been shown to enhance brain and physical functioning while chronic stress can often lead to severe illnesses, both behavioural and physical[2]. A stressor is defined as a physical and or psychological stimulus that disturbs homeostasis and activates a stress response aimed at restoring a state of balance while preparing for potential future stressors. In case of persistent or chronic exposure to a stressor the adaptive responses of an organism can become exhausted, creating a new non-functional balance[3-10], which has been linked to increased risk for a range of stress-related disorders (SRDs) such as major depressive disorder (MDD) and post-traumatic stress disorder (PTSD)[11]. The group of SRDs thus refers to disorders that can be characterized by maladaptive responses to traumatic or stressful event(s) in a given period of time[12].

While evidence supports a strong role for exposures to chronic or severe stress and/or trauma in the aetiopathogenesis of psychiatric and physical disorders, it has also been noted that not all individuals will suffer the consequences of chronic stress. Instead, a considerable proportion of individuals show tolerance to stressful or traumatic situations. Clinically, the latter is referred to as the phenomenon of resilience, while individuals that display a maladaptive stress response are referred to as being vulnerable or susceptible to stress[13].

The stress response

The primary stress-response systems in mammals are the sympathetic nervous system and the hypothalamic-pituitary adrenal (HPA)-axis[14]. Glucocorticoids (GCs) are predominantly released by the HPA-axis and are key elements in the first response to a stressor as well as in the long-term physiological responses to stress (**Figure 1**)[15]. In brief, during a stressful event, parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing hormone (CRH) in the venous portal system of the pituitary. In the anterior pituitary, CRH stimulates corticotropic cells to synthesize adrenocorticotropic hormone (ACTH), which is released in the blood stream. In turn, ACTH stimulates the production and secretion of GCs, which are steroid

hormones, from the adrenal cortex[16]. In humans the main endogenous GC is cortisol (CORT) whereas in rodents it is corticosterone. The pulsatile release of GCs follows a circadian and ultradian rhythm which results in peak levels of GCs in the mornings[17]. Circulating GCs are related to a plethora of physiological processes such as energy mobilization, metabolic changes, and immune responses. During acute stress, HPA-axis activity is rapidly increased which leads to higher levels of circulating GCs[18]. Stress-induced GC levels in pathological states (between 420 and 779 nM[19]) have been shown to be several fold higher than diurnal baseline levels of circulating GC levels (between 137 and 283 nM)[20])[21, 22]. The effects of GCs are mediated by two types of steroid receptors: the glucocorticoid receptor (GR), encoded by the nuclear receptor subfamily 3 group C member 1 (*NR3C1)*, and the mineralocorticoid receptor (MR), encoded by the nuclear receptor subfamily 3 group C member 2 (*NR3C2),* with endogenous GCs harbouring higher affinity to the MR than the GR[18, 23].

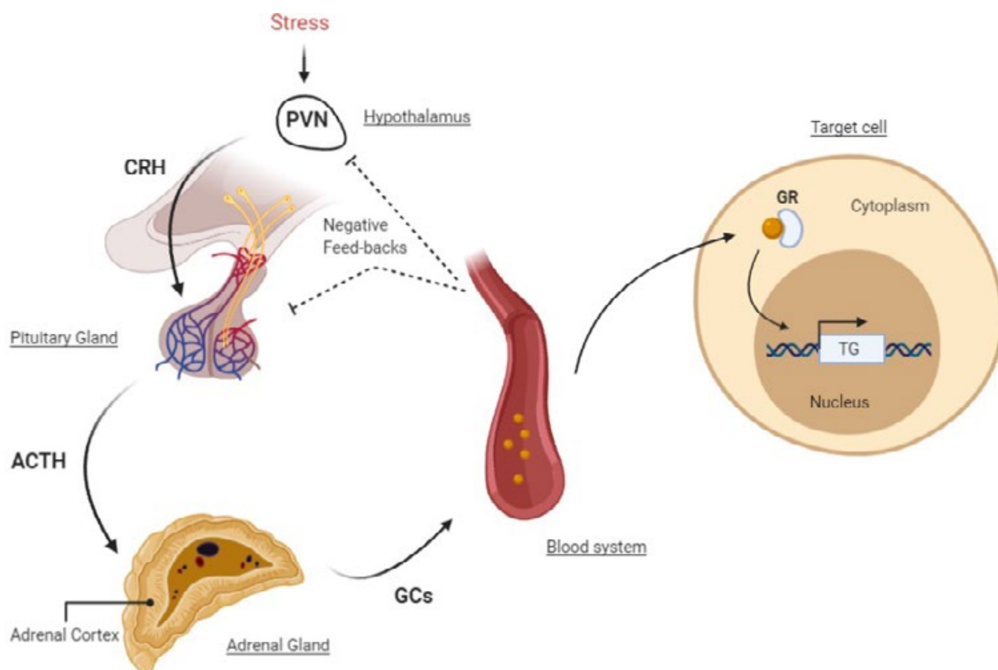


Figure 1: **Stress activation of the hypothalamic-pituitary adrenal (HPA) axis.** After exposure to a stressful situation, the activity of the HPA axis is increased. In those conditions, the paraventricular nucleus (PVN) releases corticotropin-releasing hormone (CRH). CRH then binds to its receptor in the anterior part of the pituitary gland promoting the secretion of adrenocorticotrophic hormone (ACTH) into circulation. Finally, ACTH reaches the adrenal gland and stimulates the production of glucocorticoids (GC) by the adrenal cortex of the adrenal glands. Therefore, GCs will be secreted into the bloodstream and reach diverse cells and organs in the body, leading to the transcription of target genes via activation of glucocorticoid receptors (GR). As a part of homeostatic mechanisms in the body, the HPA axis is subject to robust negative feedback inhibition by GCs. (This figure has been created with BioRender.com)

Following the binding of GCs, the receptor is activated and may induce both genomic and non-genomic pathways. Focusing on the genomic pathway, the activated receptor translocates to the nucleus and acts as transcription factor by binding to specific DNA sequences known as glucocorticoid response elements (GREs)[24]. These GREs influence the transcriptional expression of genes[25] involved in numerous physiological processes such as inflammation (acting as anti- or pro-inflammatory facilitator)[26], synaptic plasticity[27], and apoptosis[28].

Under normal circumstances, once the stressor subsides, the HPA-axis is dampened via the inhibiting effects of GCs at the level of the PVN and the pituitary. This negative-feedback mechanism relies heavily on GC-GR signalling[29]. A key player in the regulation of GR expression is FKBP prolyl isomerase 5 (*FKBP5*), acting as a co-chaperone to the GR influencing its sensitivity to GCs[30]. Increasing evidence points towards dysregulation of the neuroendocrine system in subsets of patients with PTSD[31] and MDD[32], predominantly within the HPA-axis[33, 34], even though these are not always consistent. HPA-axis dysregulation can be measured with the dexamethasone (DEX) suppression tests (DST). DEX is a synthetic glucocorticoid and selective GR agonist, that, when administered, stimulates the negative feedback loop resulting in suppression of GC release. DST studies suggest that the HPA-axis may be hypo-suppressed in MDD and hyper-suppressed in PTSD[35]. However, it remains unclear whether this HPA-axis dysregulation is a cause, consequence, mediator, or moderator in the development of SRDs[36-39]. It should also be noted that HPA-axis dysregulation is mainly reported in conditions of early life adversity, implying a neurodevelopmental context for SRD pathogenesis[40].

In vitro brain models

Given the scarcity and difficulty in the use of human brain tissue as well as the ethical implications associated with it, scientists have turned to animal and cellular models in order to better understand how GCs contribute to stress reactivity and neurobiological changes[41]. Animal models have indisputable importance for the study of the brain at physiological and disease conditions as well as in response to environmental stimuli. This review will focus on *in vitro* models used as an additional way to study aspects of brain functioning.

Despite the limitations of *in vitro* studies, they have regained attention in the past decade, especially through the advent of induced pluripotent stem cell (iPSC)-derived models, which allow the direct investigation of patient-derived cells and disease-specific phenotypes. These models are now being considered as one of the pivotal pillars of contemporary neurobiology research due to their numerous advantages. In addition to the possibility of generating cells of human origin, other advantages of iPSC-derived

models include the potential for straightforward drug testing, genetic and epigenetic manipulations, and relatively lower costs than *in vivo* experiments. Moreover, the need for robust *in vitro* model systems is warranted by increasing international efforts founded on the 3R principle (Refining, Reducing, and Replacing animal models) for animal research[42]. Therefore, combining *in vivo* and *in vitro* studies to explore certain mechanisms is vital.

A variety of *in vitro* neuronal models have been used to investigate the effects of GCs on neuronal processes. These range from animal primary neuronal cultures, *ex-vivo* brain slices, animal, or human neuroblastoma cell lines (e.g., SH-SY5Y cells) and embryonic stem cell (ESC)- or iPSC-derived neuronal models. These include both 2-dimensional (2D) cultures and 3-dimensional (3D) organoid cultures that model certain brain regions, such as the cortex or the hippocampus (**Figure 2**). The efforts to model more than one brain region are now focusing on combining organoids of different regions in one structure called assembloids[43-46]. Each of these models can be used to answer specific research questions and each carries its unique advantages and disadvantages (**Figure 3**). For

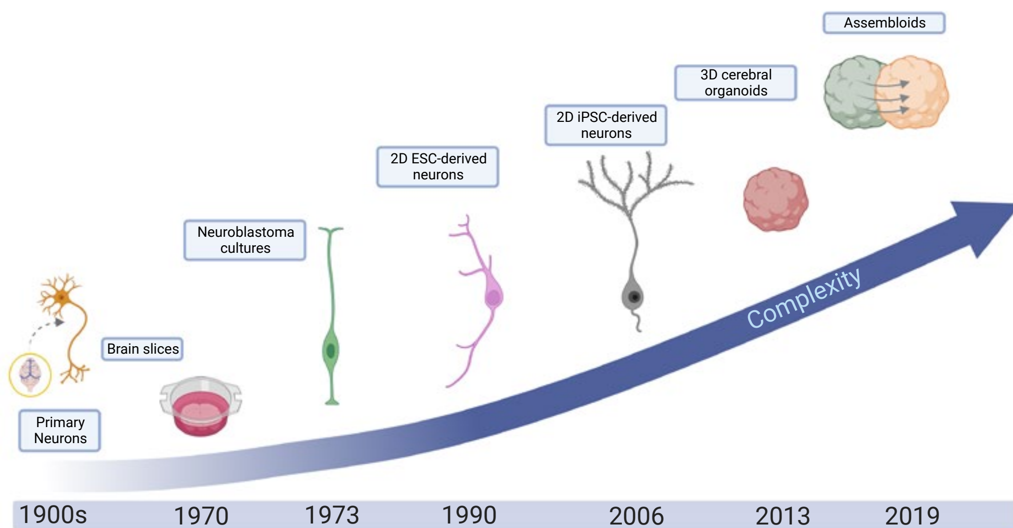
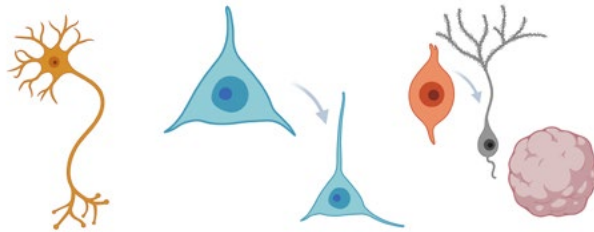


Figure 2: Evolution of *in vitro* brain models used for investigating effects of glucocorticoids. Schematic representation of past and emerging in vitro neuronal models with increasing resemblance to human in vivo brain functioning, that have been used for the investigation of the neurobiological effects of glucocorticoids. These models include primary neurons, brain slices and neuronal networks (e.g., organotypic slice cultures), neuroblastoma cultures, 2D pluripotent-stem cell-derived (PSC) neurons, 3D organoids of different brain-regions and assembloids. (This figure has been created with BioRender.com)

instance, primary neuronal cultures and *ex-vivo* brain slices maintain high fidelity to *in vivo* biology but are a less abundant resource. Neuroblastoma cell lines hold a relatively lower cost compared to primary cultures and carry human-specific biology which can be missing in rodent cultures. Additionally, they can be used both in their immature-undifferentiated stage as well as at a more mature-differentiation stage[47]. On the other hand, these cells are cancerous and have been genetically modified to induce stable, proliferating cultures and do not recapitulate the physiological proliferation, maturation, and death cycles of neuronal



Property (investigated to date)	Primary Cells	Neuroblastoma	PSC-derived neural cultures (NPCs and Neurons)
Cell sources	Brain regions (cortex, hippocampus, amygdala, hypothalamus, glial cells)	Brain Tumors	Fibroblasts, blood cells
Species	Animal or Human	Human	Animal or Human
Can differentiate further?	Yes	Yes	Yes
Time in culture	Days to Weeks	Days	Weeks to Months
Costs	Low	Low	High
Epigenetic status	Available (donor-specific in the case of post-mortem samples)	Available	Available (donor-specific)
Genetic analysis	Genotype-specific (in the case of GMO) or donor-specific (in the case of post-mortem samples)	No	Yes (donor-specific)
Capacity to model neurodevelopment?	Yes (if taken at early neurodevelopmental stages in animals)	No	Yes
Immortalized?	Possible	Possible	No
Complexity and Diversity			Complex 3D cultures can be generated
Mature markers	Yes (if taken at adult stages)	Undifferentiated: no; Differentiated: yes	Possible

Abbreviations: PSC, pluripotent stem cells; NPC, neural progenitor cells; GMO, genetically modified organism.

Figure 3: Comparing different *in vitro* brain models used for investigating neurobiological effects of glucocorticoids. (Images have been created with BioRender.com)

This review outlines recent findings on some of the molecular and cellular mechanisms underlying GC effects *in vitro*, which can provide some evidence for mechanisms involved in susceptibility to SRDs[48, 49]. We do acknowledge that the neurobiology of stress does not rely solely on the effects of GCs, and that GC exposure does not translate to stress exposure *in vitro[50]*. For instance, noradrenaline, CRH, and other stress-related hormones all play a critical role in the stress response. And the effects of GCs only partially explain the stress response and its effects on cells in the central nervous system (CNS) and the development of SRDs. Additionally, inducing cellular stress mechanisms *in vitro* can be performed beyond treating cells with GCs, and that includes models of oxidative stress, nutrient deprivation, heat shock, treatment with chemicals (e.g., toxins), and mechanical stress, among others[51]. However, as the literature on this topic is quite expansive in relation to SRDs, we provide an overview of a selected number of critical landmark studies (as opposed to providing a systematic review of the available literature). We start by mentioning limitations and challenges within the field such as the difficulty of identifying and optimizing experimental conditions and outcome parameters to differentiate between adaptive (allostasis) and maladaptive (allostatic load) responses, and the differential effects of acute versus chronic stress *in vitro*. We review studies that make use of GCs (namely CORT; corticosterone in animals or cortisol and hydrocortisone in humans, and DEX) because of their key role in the stress response and in stress susceptibility[52]. We focus on some of the most commonly used *in vitro* models and approaches the field is advancing. We begin by highlighting findings involving genetic liability/moderation and epigenetic changes following GC exposure. We then discuss GC-induced effects on molecular and cellular processes including neurogenesis, synaptic plasticity, and neurotoxicity among others. We end by highlighting studies looking into the effects of GCs on neurotransmitter systems and glial cells. The findings of the studies are described and summarized in **Table 1**, together with an overview highlighting some key findings in **Figure 4**. Finally, we provide future perspectives on the importance of developing better *in vitro* models for investigating the neurobiological effects of GCs.

Table 1 *In vitro* studies examining the role of glucocorticoids in central nervous system cell lines.

a. Genetic and epigenetic variations underlying GC effects

Publication	Cell line/model	GC tested	Concentration(s) used	Exposure duration	Primary finding
[61]Lieberman et al. (2017)	Human iPSC-derived neurons	DEX	1 µM	6 hours	DEX exposure leads to an increase in FKBP5 mRNA expression regardless of the FKBP5 genotype.
[100]Nold et al. (2020)	Primary mouse astrocytes, microglia, and (cortical and hippocampal) neurons	DEX or CORT	0.8, 4, 20, and 100 nM	4 hours	Astrocytes, microglia, and neurons exhibit differential *FKBP5* expression in response to GCs, with astrocytes being the most responsive. These results further highlight the role of astrocytes in the stress response and *FKBP5*-associated functions.
[63]Seah et al. (2022)	Human iPSC-derived mixed forebrain neurons and induced-NGN2 neurons	DEX and HDC	100, 1000, and 2500 nM	6 and 24 hours	GC exposure produces cell-type specific stress responses and concentration-dependent differential genetic expression that could be used as a diagnostic tool for PTSD risk.
[99]Hay et al. (2014)	Primary neonate neurons	DEX	50 µM	16 hours	SNPGR, is a DEX response element of the TAC1 (gene encoding substance-P) promoter region which leads to an increased promoter activity if carrying the T-allele.
	SH-SY5Y neuroblastoma	DEX	50 µM	24 hours	SNPGR, is a DEX response element of the TAC1 (gene encoding substance-P) promoter region which leads to an increased promoter activity if carrying the T-allele.
[111]Bose et al. (2010)	Sprague Dawley Rat embryonic neural stem cells [E15]	DEX	1 µM	48 hours	DEX exposure reduces proliferation of NSC, upregulates genes associated with cellular senescence, and downregulates genes related to mitochondrial functions, possibly due to changes in gene methylation and leading to increased vulnerability to oxidative stress in daughter cells.
[112]Bose et al. (2015)	Sprague-Dawley rat primary cortical neural stem cell cultures [E15]	DEX	1 µM	48 hours	DEX exposure led to a genome wide hypomethylation associated with a decrease in Dnmt3a and an increase in Dkk1 via an increase in Tet3 expression.
[113]Provençal et al. (2019)	Hippocampal progenitor cells and neurons	DEX	1 µM	3 or 10 days	Changes in DNAm and RNA expression followed DEX exposure. These changes were enhanced at human brain fetal development stages. Long lasting DMSs correlated with a second acute GC exposure.
[115]Lee et al. (2010)	Mouse HT22 hippocampal neurons	CORT	1 µM	6 hours, 1; 3; 5; 7 days (with and without washout for 7 days)	Following chronic CORT exposure an increase in FKBP5 mRNA expression was accompanied by a decrease in DNA methylation.

Table 1 continued

b. Molecular underpinnings of GC effects

Publication	Cell line/model	GC tested	Concentration(s) used	Exposure duration	Primary finding
[119]Verjee et al. (2018)	SH-SY5Y neuroblastoma	DEX	10 µM	6 or 48 hours	Both short and long DEX exposure led to an increase in FKBP5 and NET expression, and a decrease in CREB, GRIK4, VEGF, ARRB2 expression.
[120]Sabbagh et al. (2018)	Mice ex vivo slice cultures and wild-type primary neurons	DEX	100 nM and 0.5 µM	3 and 4 hours	Benztropine increases glucocorticoid-induced GR nuclear translocation in the presence of high levels of FKBP5.
	M17 neuroblastoma	HDC	50 nM	16 hours	CORT induces GR activity and to a lesser extent in the presence of an FKBP5 vector.
[122]Karst et al. (2000)	Mouse CA1 pyramidal neurons	CORT	100 nM	20 min	Exposure of hippocampal neurons to GCs is initiated by the homodimerization, translocation, and GR binding to DNA as seen by an increase in peak and sustained calcium current amplitude.
[123]Cote-Vélez et al. (2008)	Primary hypothalamic cultures	DEX	10 nM	1 or 3 hours	DEX exposure leads to an increase in mRNA expression of TRH upon binding to the GR, through the activity of PKC and ERK signaling.
	SH-SY5Y neuroblastoma	DEX	10 nM	1 or 3 hours	
[126] Díaz-Gallardo et al. (2010)	Primary hypothalamic cultures	DEX or CORT	10 nM or 100 nM	1 hour	Cells treated with GCs reveal that several transcription factors including p-CREB, c-Jun, and c-Fos bind to the TRH promoter. This effect was antagonized in the presence of cAMP.
[127] Pérez-Martínez et al. (1998)	Primary rat hypothalamic cell cultures	DEX	10nM – 10mM	1-3 hours	DEX regulates the expression of *TRH* in a dose-dependent manner, while low and high concentrations inhibit or reduce its expression, intermediate doses provoke an enhanced *TRH* expression.
[128]Cote-Vélez et al. (2005)	Primary hypothalamic cell cultures	DEX	10 nM	1 or 3 hours	DEX exposure provokes interference on the cAMP pathway and upregulates *TRH* expression via CRE and GRE at a transcriptional level.
	SH-SY5Y neuroblastoma	DEX	10 nM	1 hour	
[133] Jeanneteau, Garabedian, and Chao. (2008)	Rat cortical brain slices [P9 and P10]	DEX	1 µM	0.25; 0.5; 2; 4; or 6 hours	GCs enhance the activation of TrkB receptor independent of neurotrophins resulting in neuroprotective effects.
	Rat cortical neurons (deprived of B27 for 5 hours)	CORT	1 µM	3 hours	
	Rat cortical and hippocampal neurons	DEX	1 µM	4 hours	

Table 1 continued

[134] Kumamaru et al. (2008)	Rat hippocampal neurons [P2]	DEX	0.1; 1;10; 100 µM	3 days	In immature neurons, DEX exposure led to a decrease in BDNF-stimulated dendritic outgrowth and levels of synaptic proteins. In mature neurons, DEX led to a decrease in BDNF-induced postsynaptic calcium influx and presynaptic glutamate release.
[135] Kumamaru et al. (2011)	Rat cortical neurons [P2]	DEX	0.01-10 µM	4 days	DEX inhibits Sph2-TrkB interaction possibly via suppression of ERK signaling.
[136]Pandya et al. (2014)	Primary cortical neurons	CORT	1 µM	3 or 48 hours	Acute GC exposure upregulates the TrkB receptor via activation of the GR in young neurons only. While chronic GC exposure downregulates TrkB expression in both young and mature neurons.
[137] Numakawa et al. (2009)	Rat cortical neurons	DEX and CORT	1 µM	24 or 48 hours	DEX and CORT chronic exposure decreased BDNF-mediated release of glutamate via suppression of PLC-γ/Ca^{2+} signaling. Additionally, TrkB-GR interaction was reduced due to a decrease in GR expression.
[139]Gite et al. (2019)	SH-SY5Y neuroblastoma	CORT	500 µM	24 hours	CORT exposure decreased viability of neurons, and mRNA expression of BDNF-VI and CREB1.

c. Cellular processes underlying GC effects

Publication	Cell line/model	GC tested	Concentration(s) used	Exposure duration	Primary finding
[56]Cruceanu et al. (2020)	Human induced pluripotent stem cell (iPSC)-derived cerebral organoids	DEX	10, 100, and 1000 nM and 100 µM	4 and 12 hours	DEX exposure show delayed transcript regulation of differentiation and maturation processes due to GR activity. DEX exposed neurons also display differential expression in genes associated with behavioral phenotypes and disorders.
[60]Anacker et al. (2013)	Immortalized human hippocampal progenitor cell line HPC03A/07	CORT	100 nM and 100 µM	3 days	Low CORT concentrations increased proliferation of progenitor cells and differentiation into astrocytes, and decreased neurogenesis via MR activation. High CORT concentrations decreased proliferation and neurogenesis via GR activation.
[80]Karst et al. (2005)	Mice hippocampal slices	CORT	1-100 nM	0-5 min or 5-10 min or 2.5-50 min	CORT rapidly increases mEPSC frequencies in hippocampal cultures and decreases paired-pulse facilitation. This GC rapid effect is mediated mainly via the MR.
[82]Munier et al. (2012)	Murine human embryonic stem cell-derived neurons	CORT	100 nM	6 hours	CORT had differential effects on the Bcl2/Bax ratio in wild-type neurons and neurons with overexpressed MR, with the Bcl2/Bax ratio being substantially increased in the MR-overexpressed neurons.
[143]Nürnberg et al. (2018)	Human iPSC-derived neural progenitor cells and neurons	DEX	5; 50; 500 nM 50 nM	7, 14, 28, 50 days	DEX exposure leads to an increase in NPC proliferation and a decrease in neuronal differentiation mediated via the GR. The enzyme 11-β-hydroxylase CYP11B1 involved in GC synthesis was expressed in both NPCs and neurons.

Table 1 continued

[144]Ninomiya et al. (2014)	iPSC-derived neural progenitor cells and neurons	DEX, betha-metha-sone, and HDC	5 and 500 nM and 50 µM	4 days	Different GCs led to an increase in NPC proliferation with increasing concentrations. An increase in MAP2+ neurons was also observed. Under oxidative stress conditions, HDC only led to an increase in MAP2+ neurons.
[146] Abdanipour et al. (2015)	Rat neural stem/precursor cells from sub-granular and sub-ventricular zones	CORT	0; 0.25; 0.5; 1; 2.5; 5; 10; 15; 20 µM	24; 48; 72; 96; 120 hours	High concentrations of cortisol have anti-proliferative effects on NSCs in a dose- and time-dependent way via apoptosis and necrosis.
[147]Yao et al. (2007)	Primary rat embryonic hippocampal neurons	DEX	0.01; 0.1; 1; 10 µM	48 hours	Exposure to DEX increases susceptibility to the effects of amyloid-β, increases intracellular calcium concentrations, and reduced the amyloid-β-induced expression of NF-κB p65 proteins.
[183]Koo et al (2010)	Rat adult hippocampal progenitor cells	CORT	10 µM	2 hours	CORT negatively affected proliferation of cells with no influence on cell death, This effect is mediated by p39 MAPK signaling and the GR.
[148]Behl et al. (1995)	Mouse HT22 hippocampal neurons	CORT	100 µM	20 hours	Exposure to GCs did not show neuroprotective effects in the presence of neurotoxins, leading to a substantial decrease in cell survival.
[149]Anacker et al. (2013)	Human hippocampal progenitor cell line HPC03A/07	CORT	100 µM	1, 3, 12, or 72 hours	CORT reduces hippocampal progenitor cell proliferation and differentiation via an increase of SGK1 expression. Inhibition of Hedgehog signaling and increase of GR function are mediated by SGK1.
[150]Kim et al. (2004)	Adult rat hippocampal progenitor cells.	DEX	5 µM	12 hours	DEX exposure inhibits proliferation of progenitor cells, enhances p21 expression, and impairs ERK activation and SRE activity.
[151]Yu et al. (2004)	Primary rat fetal hippocampal progenitors	CORT and DEX	2, 20, 200 nM / 2, 5, 20, 40, 50 µM	3 days	CORT reduces cell proliferation alters NeuroD, BDNF, and NR1 expression, and provokes dendritic atrophy in a dose-dependent manner.
[153] Crochemore et al. (2005)	Primary hippocampal rat neurons	DEX	1 and 10 µM	48 hours	DEX provokes neuronal cell death via GR-mediated apoptosis
[154]Tamura et al. (2005)	SH-SY5Y neuroblastoma	CORT	0.6 mM	1; 3; 6; 12; 24 hours	CORT exposure decreases Tll-1 promoter activity and Tll-1 mRNA expression.
[159]Anacker et al. (2011)	Human hippocampal progenitor cell line HPC03A/07	DEX and CORT	1 µM DEX and 100µM CORT	72 hours, 7 days, and 10 days	Antidepressant reverses GC-induced decrease in proliferation and neurogenesis via GR-mechanisms involving PKA signaling, GR phosphorylation, and upregulation of GADD45B, SGK1, and FOXO1 expression.
[160]Xi et al. (2011)	Primary rat hippocampal neural stem cells	DEX	0.01, 0.1, 0.5, and 1 µM	48 hours	Antidepressants reverse DEX-inducing upregulation of TREK-1 and reduction in NSC proliferation.
[161]Yeo et al. (2019)	SH-SY5Y neuroblastoma and human ESC-derived neural stem cells and neurons	DEX	1;10;100;250; 750;1000 µg/mL (between 2 µM and 2 mM)	48 hours	DEX led to a decrease in cell viability via an increase in apoptosis, and a decrease in pAkt levels.

Table 1 continued

[169]Pu et al. (2007)	Rat brain slices	CORT	100 nM	15 or 20 min	CORT differentially regulates beta-adrenergic associated synaptic plasticity, depending on the timing of administration.
[170]Jafari et al. (2012)	Mice adult hippocampal slices	DEX	5 µM	5, 15, or 30 minutes	DEX exposure modulates synaptic plasticity via alterations in p-Cofilin levels, ERK1/2, number of PSD95+ spines, and pCofilin immunoreactive spines.
	Sprague Dawley rat cultured hippocampal slices	DEX	5 µM	15 min to 1 hour	
[174]Bhargava et al. (2002)	Rat hippocampal H19-7 neurons	CORT	100 nM	30; 60; 120 min	CORT leads to an extended increase in intracellular calcium concentrations via the inhibition of PMCA1.
[176] Suwanjang et al. (2013)	Primary Sprague-Dawley rat cortical midbrain, and hippocampal neurons, and astrocytes	Dexa-metha-sone and corti-coste-rone	1 µM	3-5 min	Brief exposure to GCs reduces basal levels of cytosolic calcium concentrations in both neurons and astrocytes via the GR and independent of the NMDAR, without showing signs of toxicity. These results suggest that GCs are used for the protection of neurons from glutamate cytotoxicity.
[177]Chen et al. (2011)	Wistar rat hypothalamic primary neuronal slices	DEX	10 µM	Within seconds to min	Rapid effects of DEX led to a decrease in intracellular calcium concentrations in primary rat hypothalamic neurons. This is suggested to be mediated via GR and plasma membrane calcium pumps activation.
[179]Du et al. (2009)	Rat primary cortica neurons [E18]	CORT	100 nM, 500 nM, and 1 µM	Ranging between 0 and 72 hours	High CORT levels lead to kainic acid induced toxicity and changes in mitochondrial function in cortical neurons, partly via a decrease in GR/Bcl-2 levels in the mitochondria.
[180]Luo et al. (2020)	Sprague-Dawley rat primary cortical neurons [E18]	CORT	100 nM and 1 µM	30 min, 24 hours or 3 days	CORT exposure regulates the formation of GR/Bag-1 complex in a dose and time-dependent manner in rat primary cortical neurons. Prolonged exposure led to a negative regulation of the complex and a reduction in mitochondrial GR levels.
[184]Zhu et al. (2018)	Mice hippocampal primary neurons (7DIV)	CORT	10 µM	24, 48, or 72 hours	GCs significantly increase levels of NF-κB subunits, activating NF-κB signaling.
[185]Bharti et al. (2018)	Mouse HT22 hippocampal neurons and primary cortical neurons	CORT	0.5; 1; 2 µM	5 days	Chronic CORT exposure leads to an increase in the Txnip protein expression in both the nucleus and cytosol by activation of the GR. Txnip was also shown to enhance protein nitrosylation and sulfenylation contributing to oxidative damage.
[186]Seo et al. (2012)	Mouse HT22 hippocampal neurons	CORT	200, 400, or 800 ng/mL	24 hours	CORT exposure leads to an increase in superoxide levels by upregulating NAPDH oxidase.

Table 1 continued

	SH-SY5Y neuroblastoma	CORT	400, or 800 ng/mL	For 2 hours daily between 1 and 3 days or 24, 48, 72 hours	CORT exposure leads to an increase in superoxide levels by upregulating NAPDH oxidase.
[188]Iqbal, Howard, and LoGrasso. (2015)	SH-SY5Y neuroblastoma	DEX	10 μM	24 hours	DEX decreases cell viability and increases endogenous SGK1 expression which carries neuroprotective effects on ROS, mitochondrial dysfunction, and cell death.
[189]Kim et al. (2018)	SK-N-SH neuroblastoma	CORT	0.25 mM	1 hour	CORT exposure decreases cell viability, ATP levels, MMP, gene expression of CREB and BDNF. To the contrary CORT increases ROS levels, caspase-3/7 activity, and pro-inflammatory cytokines.
[190]Golde et al. (2003)	Male Sprague-Dawley rats cortical cultures [E16] and primary microglia and N9 murine microglia cell line	DEX	1; 10; 100; 1000 nM	3 days	DEX exposure leads to the alleviation of neurotoxicity by decreasing NO synthesis and a reduction in iNOS mRNA and protein levels.

d. GC effects on glial cells

Publication	Cell line/model	GC tested	Concentration(s) used	Exposure duration	Primary finding
[194]Snijders et al. (2020)	Primary human microglia from post-mortem brain tissue	DEX	1 μM	72 hours	DEX exposure promotes the expression of CD163, CD200R and MRC1 in microglia. These changes observed are not different between healthy and MDD patients.
[195]Melief et al. (2012)	Primary human microglia from post-mortem brain tissue	DEX	2 nM	72 hours	DEX exposure leads to morphological changes in microglia and upregulates CCL18, CD163, and the mannose receptor.
[196]Unemura et al. (2012)	Rat primary cortical astrocyte monoculture	CORT and DEX	0.01; 0.1; 1 μM	1-6; >12; 24h; and 72 hours	GC exposure impairs astrocyte proliferation but not cell death due to GR downregulation via GR activation.
[197]Crossin et al. (1997)	Rat primary cortical astrocytes	DEX, CORT, HDC	0.1 - 10 nM; 0.01 and 1 μM	6 hours	GCs impairs astrocyte proliferation in a concentration-dependent fashion.
[198]Virgin et al. (1991)	Rat primary hippocampal astrocytes and secondary hippocampal, cortical, and cerebellar astrocytes	CORT, and DEX	1, 10, 100 nM; 1, 10 μM	24 hours	CORT exposure causes an inhibitory dose-dependent effect on glucose transport and increases sensitivity to hypoglycemia, particularly in hippocampal cells.
[199]Heard et al. (2021)	hiPSC-derived astrocytes	CORT	5, 50 μM	24 hours or 7 days	Chronic exposure to CORT resulted in MDD-specific differentially expressed genes associated with GPCR-ligand binding, synaptic signaling, and ion homeostasis in astrocytes.

Table 1 continued

[200]Miguel-Hidalgo et al. (2019)	Rat embryonic myelination neural cultures [E16] and mixed glial rat brain cerebral cortex[P1]	CORT	5, 50µM	4 days (with and without replen-ishing) or 16 days (replen-ishing every 3 days)	Chronic exposure to GCs decreases myelination index, MBP and Cx43 in spinal cord and cerebral cortex myelination cultures, that is dose-dependent, mediated by the GR. Additionally, chronic glucocorticoids reduce oligodendrocyte processes.

e. **GC effects on neurotransmitter systems**

Publication	Cell line/model	GC tested	Concentra-tion(s) used	Exposure duration	Primary finding
[204]Groc et al. (2008)	Sprague-Dawley rat hippocampal neurons [E18]	CORT	10, 50, 100 nM	1-20 min; 150 min (with washout)	CORT increases hippocampal glutamate transmission in a time-dependent fashion via upregulation of the surface synaptic protein GluR2.
[205]Zhou et al. (2012)	Rat hippocampal primary cultures	CORT	30nM	15min	CORT in combination with a β-adrenergic receptor agonist regulate AMPAR phospho-rylation, surface expression, and mEPSC.
[206]Mahmoud and Amer. (2014)	Young rat hippocampal slices/tissue	CORT	0.5; 5; or 30nM	1 or 2 hours	Brief exposure to CORT is shown to increase synaptic transmission and decrease the NMDAR subunit NR2B and NR2B:NR2A ratio.
[79]Fan et al. (2018)	SH-SY5Y neuroblastoma	CORT	5 µM	3 days	CORT exposure led to an increase in Phox2a and Phox2b via GR activation.
[211]Pu et al. (2009)	Rat brain slices from the basolateral amygdala	CORT	100 nM	20 min – 2 hours	CORT slowly inhibits synaptic potentiation activated by noradrenergic effects through the β-adrenergic receptor, preventing the system from enhanced activation.
[213]Wong et al. (2015)	SH-SY5Y neuroblastoma	DEX	100 nM	24 hours	DEX exposure upregulates the expression and catalytic activity of MAO A.
[220]Tazik et al. (2009)	SH-SY5Y neuroblastoma and glioblastoma 1242-MG cells	DEX	10 µM	Every other day for 4 days	DEX exposure impairs cell proliferation and increases the activity of MAO B promoting cell death which could be prevented by antidepressant drugs or MAO inhibitors.
[221]Johnson et al. (2010)	SH-SY5Y neuroblastoma	DEX	2 µM	Daily for 3 days	DEX exposure provokes an increase in the catalytic activity of MAO enzymes leading to cell death and DNA damage, these effects can be counteracted or reduced by MAO inhibitors like M30.

Abbreviations: CORT: cortisol or corticosterone; CRE: cAMP response element; DEX: dexamethasone; DIV: days in vitro; DMS: differentially methylated sites; GC: glucocorticoid; GR: glucocorticoid receptor; GRE: glucocorticoid response element; HDC: hydrocortisone; iPSC: induced-pluripotent stem cell; iNOS: inducible nitric oxide synthase; MAO(-B): monoamine oxidase (-B); MDD: major depressive disorder; MR: mineralocorticoid receptor; NA: noradrenaline; NMDAR: N-methyl-D-aspartate receptors; NO: nitric oxide; NPC: neural progenitor cell; NSC: neural stem cell; PLC-γ: Phospholipase C Gamma; PTSD: port-traumatic stress disorder; ROS: reactive oxygen species; SER: serum response element; TrkB: Tropomyosin receptor kinase B.

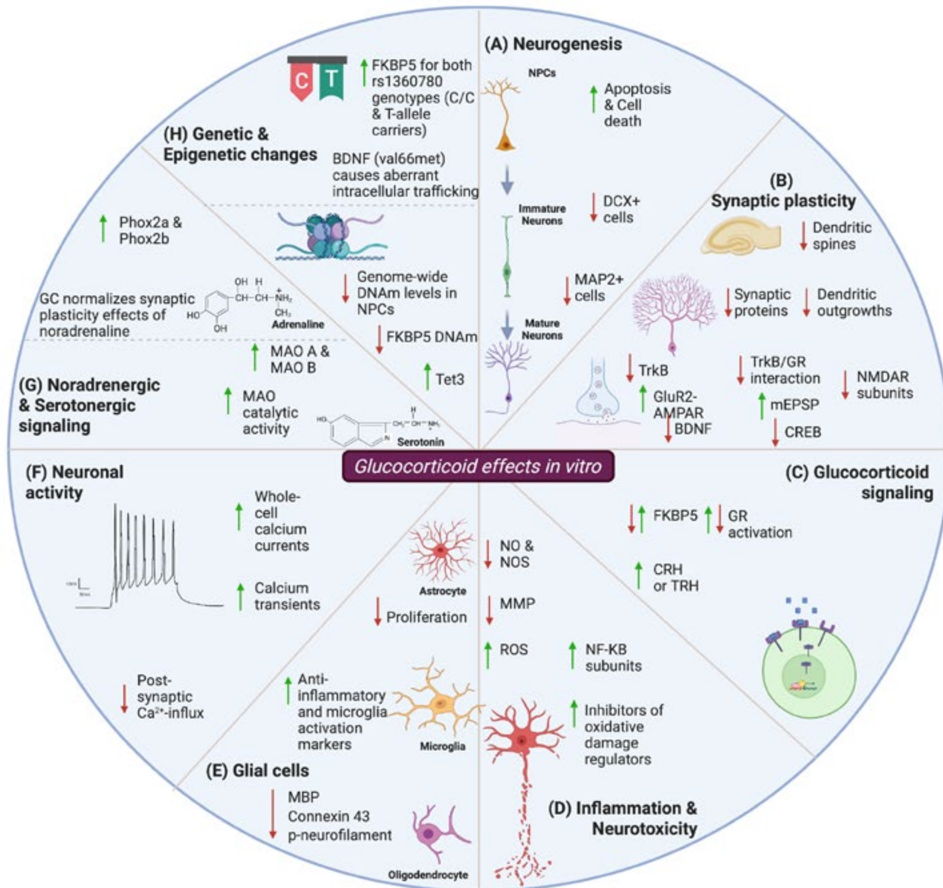


Figure 4: Key findings of studies investigating glucocorticoid's neurobiological effects *in vitro*. Exposure to GCs affects many neurobiological aspects, including neurogenesis, GC signalling, inflammation and toxicity, myelination, synaptic plasticity, physiological activity, and genetic and epigenetic mechanisms. (A) GCs impact neurogenesis by having an effect on neural progenitor proliferation and survival, and decreasing the process of generating new neurons. (B) GCs negatively impacts synaptic plasticity particularly in hippocampal neurons by downregulating essential synaptic proteins, dendritic spines and outgrowths. (C) GCs alter glucocorticoid signaling and result in a downregulation of GR activity and translocation. (D) GC exposure exhibits both anti- and pro-inflammatory properties with the latter leading to an increase in neurotoxicity markers such as reactive oxygen species. (E) GCs alter glial functioning by decreasing the levels of myelin-associated proteins, proliferation of astrocytes, and increasing microglia activation markers. (F) GCs alter neuronal activity as seen with increases in calcium transients and currents. (G) Changes in noradrenergic and serotonergic signaling following GC administration. (H) Changes in epigenetic mechanisms, particularly DNA methylation, has been observed following GC exposure, possibly impacting the function of several regulatory genes, such as FKBP5. GC exposure leads to an increase in the GR regulator *FKBP5* in individuals carrying the *FKBP5* variant rs1360780. Abbreviations: BDNF, brain-derived neurotrophic factor; CRH, corticotrophin-releasing hormone; CREB, cAMP response element-binding protein; DCX, doublecortin; FKBP5, FK506 binding protein 5; GC, glucocorticoid; GluR2-AMPAR, GluR2 subunit-AMPA receptor; GR, glucocorticoid receptor; MAP2, microtubule-associated protein 2; mEPSP, miniature excitatory postsynaptic potential; MBP, myelin binding protein; MMP, mitochondrial membrane potential; MAO monoamine oxidase; NPCs, neural progenitor cells; NF-kB, nuclear factor kappa B; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; NMDAR, N-methyl-D-aspartate receptor; TRH, thyrotropin-releasing hormone; TrkB, tropomyosin receptor kinase B. (This figure has been created with BioRender.com).

Considerations for GC experiments in vitro

In vitro experiments investigating the effects of GCs on neuronal cultures (overview can be found in **Table 1**, and **Figure 4**) even though promising, are associated with a number of challenges and limitations. These include a lack of standardized protocols for acute and chronic GC exposure, a broad range of GC concentrations investigated, variability between *in vitro* models, and lack of standardized assessments for GC-induced phenotypes. It is important to consider these aspects when replicating or designing new experiments[53].

In vitro definitions of stress

There is a lack of consensus regarding key terminologies such as acute versus chronic and short-term versus long-term effects, which makes it difficult to compare results and interpret them. To improve reproducibility, it is suggested to make use of established acute and chronic stress paradigms in animal stress experiments, tailored to specific types of models (2D versus 3D). For instance, while a 3-day GC exposure in some 2D-neuronal cultures such as induced neurons, can be considered chronic exposure, this duration is not sufficient to investigate chronic effects in cerebral organoids, which have prolonged time windows.

Sources of variability

Sources of variability in GC *in vitro* studies include highly variable concentrations, differences in differentiation protocols and *in vitro* models. GC concentrations used *in vitro* range from as low as 10nM to 2mM, including concentrations that do not resemble *in vivo* concentrations. It remains a challenge to accurately measure CORT levels immediately after experiencing a stressor in humans[54], even in situations where a better estimation can be made, such as maternal stress[55] and pregnancy (see supplementary information in[56]). Importantly, GCs are also known to bind to the plastic of the culture dish[57] and have different half-lives among different types of GCs[58], meaning the final effective concentrations may vary. Interestingly, despite making use of high concentrations (e.g., 1 µM or higher), *in vitro* GC exposure often does not seem to exert profound neuronal effects. This observation could be due to the fact that *in vitro* neuronal cultures are supplemented with a variety of growth and neurotrophic factors, including serum (e.g., SH-SY5Y cultures) or supplements such as B27 (e.g., PSC-derived neurons) which already contains steroids essential for proper neuronal growth and maturation[59]. The presence of GCs in neuronal media ensures neuronal survival in-a-dish which could minimize the effects of exposure to GCs *in vitro*, hence requiring higher concentrations of GCs to ensure sufficient downstream effects of GR activation[60]. However, this could also be due to the lack of functional GR/MRs in some neuronal cell models[61] (see

Figure 3 for comparison between models). Differences in culture and differentiation protocols among *in vitro* models, and specifically in reprogramming and differentiation protocols within PSCs (transdifferentiation versus indirect conversion)[62] are also major sources of variation (some more than others) that could influence the GC-induced phenotype (as seen in[63]). Nevertheless as showcased in **Figure 3**, each model (and specifically differentiation protocols) carry advantages and limitations that speak to unique research questions and should be taken into consideration in the selection of the model[53]. Given that the generation of iPSCs from donors retain the genotype and in some instances even traces of the epigenotype, iPSC-based models can be a promising for investigating gene-environment interactions[64], especially that SRDs cannot be explained by underlying genetic vulnerability alone. This model also has the advantage that a variety of neuronal subtypes can be generated (i.e., dopaminergic, serotonergic and cortical neurons[65-67]), allowing for region-specific GC-induced phenotype identification since maladaptive changes induced by chronic GC exposure in the prefrontal cortex and hippocampus, for instance, reflect an opposite trend as compared to the amygdala[68].

Assessment of GC-induced phenotypes

It remains challenging to identify objective and standardized readouts to characterize distinct GC-induced phenotypes. One constant readout that all studies investigating GC effects could include is measuring whether GC treatment does activate the GR and/or MR. For example, measuring the expression of known GC-responsive genes such as FKBP5, TSC22D3, SGK1, ZBTB16, among others. Another major concern is the lack of objective biomarkers for psychiatric disorders, hence the inability to select robust cellular or molecular readouts to characterize specific disease-phenotypes *in vitro*[69, 70]. Current neurobiological models of psychiatric disorders do not capture the full range of clinical manifestations. For instance, no single biological process is present in MDD, and MDD symptoms involve neurobiological circuitries that overlap with other psychiatric disorders (e.g., PTSD)[71]. Nonetheless, a few characteristics to reflect cellular phenotypes of particular psychiatric disorders can be incorporated in *in vitro* studies, which include: cellular phenotypes must (1) match underlying biological pathways; (2) be measurable; and (3) be reversed using pharmacological interventions[69]. Promising examples include changes in dendritic morphology that can be measured *in vitro*[72], and making use of cell-type associations of key cognitive and psychiatric traits using[73].

Finally, the simplistic/reductionistic approach of *in vitro* models will always be an important limitation, as they examine changes occurring within a highly controlled, artificial environment. *In vitro* studies on specific pathways associated with SRDs cannot possibly capture the complexity of stress effects, knowing that the HPA-axis is in active

concert with other relevant stress-related processes[74]. For instance, it is important to note the discrepancy between *in vivo* stress exposure and CORT administration specifically, since changes observed *in vivo* following stress exposure (see example in[75-77]), is not the same as administering CORT (see example[78]). This observation could be attributed to the fact that the stress response does not only involve GCs but also other hormones and molecular mediators[79] such as noradrenergic signaling. Moreover, many studies investigating the effects of DEX, a GR-agonist, in neuronal cell lines cannot reflect the effects of stress or endogenous GCs due to the fact that DEX has a much higher affinity to the GR than other receptors implicated in the stress response such as the MR[80]. CORT is known to display higher affinity to the MR as compared to the GR[81], with MR activation being associated with neuroprotective effects[82], and GR activation - in the presence of high GC concentrations - exhibits harmful effects in neuronal cell types[60]. Additionally, MR and GR activation leads to both slow genomic and rapid non-genomic effects that involve a variety of pathways and signaling cascades[83, 84]. This balance between concentration and receptor binding is important in understanding stress vulnerabilities and downstream effects.

Genetic and Epigenetic Variations Underlying GC Effects

Psychiatric disorders are characterized by underlying genetic variants that in combination with environmental stimuli render an individual vulnerable to disease after exposure to factors such as stress[85]. For instance, individuals carrying the *FKBP5* rs1360780 risk variant have been documented to be at increased risk of developing psychiatric disorders including schizophrenia and PTSD[86, 87]. While genetic variations in *NR3C1, NR3C2*, *CRH*, *CRHR1*, and *BDNF* have also been shown to be involved in conferring risk to SRDs (see reviews[88-90]), *in vitro* studies in this field have primarily focused on *FKBP5*. Additionally, Arloth *et al*. (2015) demonstrated that common genetic variants associated with MDD and schizophrenia modify the transcriptional responsiveness of GR target genes[91]. Here, we will discuss these *in vitro* studies, which are summarized in **Table 1(a**).

Genetic vulnerabilities

In recent years, it has become more evident that both genetic and environmental factors interact to confer risk to psychiatric disorders[92-94]. Genetic variants, including single nucleotide polymorphisms (SNPs), are strongly associated with several psychiatric disorders[95], and alter the response of a single individual to particular substances such as drugs, and other environmental stimuli[96]. The use of *in vitro* models, and in

particular iPSCs, for the investigation of genetic vulnerability of psychiatric disorders has gained increased attention[63, 97], and can be used for understanding how genetic variants create differential cellular responses to, for instance, a GC challenge *in vitro*[98].

Looking at the influence of environmental factors in the presence of underlying genetic vulnerability, Seah *et al*. generated iPSC-derived mixed forebrain neurons and NGN2-induced neurons from combat-exposed veterans with and without PTSD[63]. Following exposure to different concentrations of DEX, differentially expressed genes were observed for each of the different concentrations in NGN2 neurons and, to a lesser extent, in mixed forebrain neurons. The GC responses on gene expression profiles were enriched for synaptic genes. This is a proof-of-principle study showcasing that the use of stem cell models may facilitate a better understanding of gene-environment interactions in SRDs.

Hay et al. investigated the binding of GR to a highly conserved response element, called 2GR, within the promoter region of the *TAC1* gene, which codes for the neuropeptide substance-P. This was done in primary rat amygdala cells and in SH-SY5Y cells following acute stimulation with DEX[99]. An increase in TAC1 was observed following DEX exposure, which was mediated via GR binding to 2GR within the *TAC1* promoter. A second relevant GR binding site was also identified and designated as SNPGR. SNPGR bears a T-allele polymorphism (found specifically in Japanese and Chinese populations) that enhances the stimulation of the substance-P promoter via the re-activation of the 2GR subunit. The findings on this polymorphism suggest a genetically underpinned vulnerability to GCs that may be involved in differential GR regulation and homeostasis in health and disease states[99], as was also shown by Arloth et al.

Not all studies were successful at demonstrating gene-environment interactions *in vitro*. The availability of iPSC technology has enabled us to investigate the effects of stress (i.e. GC exposure) on human neurons from individuals with an underlying genetic vulnerability for SRDs. One of the first studies attempting this was conducted by Lieberman *et al*. (2017) who studied changes in mRNA expression of *FKBP5* and *NR3C1* following a 6-hour DEX exposure (1 µM) to iPSC-derived cortical neurons from individuals with *FKBP5* rs1360780*C/C and *FKBP5* rs1360780*T-allele carriers. Acute DEX exposure increased mRNA expression of *FKBP5*, but not of *NR3C1*, irrespective of genotype[61]. Nold *et al*. (2020) exposed mouse primary neuronal cortical and hippocampal cultures, derived from humanized mouse strains carrying either the risk (A/T)or resilient (C/G) allele of rs1360780 of the *FKBP5* locus, to concentrations of DEX ranging from 0.8 to 100nM for a short incubation time (4 hours). While they did not find any significant changes in *NR3C1* expression between different DEX concentrations, they found dose-dependent

increases in *FKBP5* expression. Interestingly, no significant effect of the risk versus resilient rs1360780 allele were observed[100]. Despite both studies not illustrating any effect of the genotype on expression, this is not representative of the field as a whole, with gene-environment interaction being demonstrated in human studies[101, 102], and iPSC-derived models[63, 103]. Additionally, these studies highlight the importance of cell type differential responsiveness to GCs and GR-sensitivity, but also the importance of *in vitro* studies in unraveling the genetic risk underlying SRDs. This first wave of iPSC-based studies provided several novel insights into the use of *in vitro* studies to infer causation between genetic variance and mechanisms of disease, while also raising many questions which will be addressed in the discussion below.

Epigenetic mechanisms

Epigenetic dysregulation has been associated with a number of disorders including stress-related neurodevelopmental and other psychiatric disorders, as reviewed in[104-106]. Some of the long-term effects of GCs may be mediated via epigenetic changes, that are especially pertinent during certain developmental stages[107, 108]. Evidence indicates that GCs can impact epigenetic regulation in two ways: first by moderating the expression of epigenetic regulators and second by inducing epigenetic changes directly at GRE sites[109]. For example, genome-wide decreases in DNA methylation levels were observed in proliferating neural stem cell (NSC) cultures *in vitro* following exposure to DEX, which was shown to be mediated via an increased expression of *Tet3*, an enzyme essential for active demethylation in neurons and a crucial player in NSC differentiation[110].

Similarly, decreases in rat embryonic NSC proliferation and alterations in the expression of genes involved in cellular senescence (upregulation) and mitochondrial functions (downregulation) in NSCs following DEX exposure have been attributed to changes in DNA methylation. Decreases in average levels of genome-wide DNA methylation have been observed together with decreases in the levels of DNA methyltransferases (DNMTs). Interestingly, subsequent experiments indicated that these global changes in epigenetic processes conferred an increased vulnerability to other types of stress (i.e. oxidative stress) *in vitro* in daughter cells which were never directly exposed to DEX[111, 112], revealing a level of epigenetic memory due to GC effects.

Another study used a human hippocampal progenitor cell line to study the immediate and long-lasting effects of DEX on transcriptional and DNA methylation changes during proliferation and differentiation. Provençal & Arloth *et al*. showed that DEX treatment during the proliferation stage resulted in substantial transcriptional and DNA methylation changes[113]. Interestingly, DEX exposure after neuronal differentiation resulted

in very minimal changes both at the transcriptional and at the epigenetic level. In addition, the DNA methylation changes observed in neural progenitor cells (NPCs) persisted after a wash-out period to remove DEX and even primed the transcriptional responses to a future GC exposure. These results show that the progenitor stage is a critical neurodevelopmental stage in mediating GC effects and that changes in DNA methylation may persist within regulatory sites, priming transcriptional responses to future GC exposures[113]. Therefore, focusing on chronic stress alone is not sufficient in exploring the pathophysiology of SRDs, knowing that acute stress may also carry long-term effects[113, 114].

Another study by Lee *et al*. investigated the effects of chronic CORT exposure on *FKBP5* DNA methylation and gene expression. They observed that seven days after daily CORT exposure in the HT-22 mouse hippocampal cell line, *FKBP5* gene expression was increased, which was associated with a decrease in DNA methylation at intronic enhancers[115]. Thus, long-term CORT exposure may decrease methylation and increase expression of *FKBP5*, as well as attenuate GR activation and translocation to the nucleus. Similar findings were reported for DEX exposure in a human hippocampal progenitor cell line[113], i.e. increased mRNA expression and decreased DNA methylation in intronic enhancers. These studies indicate that GCs alter the epigenetic and transcriptional landscape. In addition, they demonstrate that *in vitro* neuronal cultures can be used to study these effects.

Molecular underpinnings of GC effects

The molecular mechanisms underlying GC effects are complex and involve intricate interactions between the GC receptors and various transcription factors and co-regulators. This section will provide an overview of *in vitro* studies looking into the molecular mechanisms underlying GC effects. The listed studies are summarized in **Table 1**(**b**).

Glucocorticoid signaling

Glucocorticoid-related genes

The regulation of glucocorticoid signaling is strongly impacted by molecules within the GR complex, as this receptor requires a number of (co-)chaperone proteins for proper functioning and is regulated by homodimerization[116]. One of the primary stress-responsive proteins that have been repeatedly linked to GR activity and stress is FKBP5. FKBP5 is a co-chaperone of the GR, which reduces the receptor’s affinity to GCs and its translocation to the nucleus, all features of GR resistance. It has been documented

that elevated levels of FKBP5 were associated with increased anxiety and decreased stress coping in rodents. In humans, genetic variants and epigenetic alterations leading to increased FKBP5 have been associated with a number of SRDs including MDD and PTSD[30, 117, 118]. For instance, exposure to DEX in SH-SY5Y cells led to time-dependent changes in *FKBP5* mRNA expression following short and long-term incubation[119].

The interaction of FKBP5 and the GR has been proposed as a pharmacological target for SRDs. Indeed, cell culture studies by Sabbagh *et al*. (2018) showed that pharmacological disruption of the FKBP5/GR complex led to a restoration of effects of DEX on GR activity and its translocation from the cytoplasm to the nucleus in primary neurons and M17 neuroblastoma cells. When studied in *ex vivo* brain slices of aged wild-type mice, DEX exposure led to an increased GR translocation from the cytoplasm to the nucleus. This translocation was also observed (albeit to a lesser extent) in the presence of increased FKBP5 levels[120]. The important role of this interaction has been corroborated by the effects of the selective FKBP5 antagonist SAFit2[121]. Together, these results offer a promising avenue to selectively target the FKBP5 complex as a potential therapeutic strategy.

Glucocorticoid receptor functioning

Changes in synaptic plasticity, neuronal activity, and cellular processes such as neuronal viability largely depend on the activation of the GR through GR homodimerization. In hippocampal slices of mutant GR mice, with the mutation preventing GR dimerization, Karst *et al*. showed that CORT-induced increases in calcium currents are dependent on receptor homodimerization and DNA binding.[122].

Downstream GR transcription factors are also important in driving the transcription of key genes with neuromodulatory functions. One study sought to study the effects of DEX on the transcription and synthesis of thyrotropin releasing hormone (TRH), a neuropeptide involved in energy metabolism. In primary hypothalamic cultures, an increase in mRNA expression of TRH is observed following DEX. Inhibition of the PKC and MAPK pathways reversed the DEX-induced effects on *TRH*, which was observed via transcriptional modifications and binding of the GR to composite GRE sites of the *TRH* promoter, particularly at the AP-1 site. These results suggest that PKC or MEK mediate the effects of glucocorticoid signaling on *TRH* transcription by decreasing binding abilities of GR to composite GRE's AP-1 binding site[123].

GC effects on other neuroendocrine genes

In addition to investigating direct effects of GCs on the HPA-axis alone, the use of *in vitro* studies may also facilitate studying the molecular and cellular functioning of other axes

involved in GC responses such as the hypothalamic-pituitary-thyroid (HPT)-axis. The HPT-axis has been repeatedly shown to be involved in SRDs[124, 125]. During the last decades, parts of the HPA- and HPT-axes could only be modeled separately in cell culture models. For example, a series of studies investigated the activation of transcription factors required for the transcription of CRH after GC-activation in hypothalamic neurons. Díaz-Gallardo *et al*. (2010) observed an increase in TRH mRNA expression following CORT or DEX exposure in rat primary hypothalamic cultures mediated via intracellular GR[126]. In another study, Pérez-Martinez *et al*. (1998) observed a dose-dependent-biphasic response in primary rat hypothalamic neuronal cultures shortly after exposure to DEX. These findings indicate that low concentrations of DEX (0.1 nM) suppressed TRH mRNA expression, while intermediate concentrations of DEX induced an increase of TRH mRNA, and higher levels (1μM) were associated with decreased expression. Together these results suggest rapid regulatory effects of DEX on TRH mRNA expression in hypothalamic neurons *in vitro[127]*. This and other studies[127-129] investigating GC-stimulated expression of other HPT hormones, including TRH, provide important insights into the interplay between neuroendocrine axes, such as the effects of GCs on *TRH* expression and noradrenaline in stress conditions.

Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) plays a crucial role in neuronal processes including neuronal survival and synaptic plasticity[130, 131]. These effects are initiated by the activation of the tropomyosin receptor kinase B (TrkB) receptor and its downstream signaling constituents, including phosphatidylinositol-3-kinase (PI3K), phospholipase Cγ (PLCγ), and MAPK pathways[132], eventually leading to the transcription of relevant genes necessary for survival and plasticity. There is evidence that GCs modulate BDNF signaling. For instance, one study focused on the acute neuroprotective effects of GCs (1 μM) in rodent brain slices[133] and showing that GCs activate TrkB receptors in neurons independently of neurotrophin release[133], which eventually enhanced neuronal survival. This suggests that GCs carry trophic properties by acting on TrkB receptors and induction of a non-canonical Akt signaling pathway.

Kumamaru *et al* (2008) showed that DEX exposure in young primary hippocampal neurons reduced BDNF-induced enhancing effects on synaptic plasticity as measured by outgrowth of dendrites and expression of (pre-)synaptic proteins[134]. DEX also decreased the BDNF-induced MAPK/ERK pathway, which mediates the downstream expression of BDNF-induced genes on survival and synaptic maturation. The effects of DEX on components of the MAPK/ERK pathway activation was further investigated with a focus on Src homology-2 domain containing phosphatase 2 (Shp2). Long-lasting ERK

signaling is required for the transcription of BDNF-induced synaptic proteins. Activation of this pathway requires the interaction of Shp2 – an ERK signaling mediator – with TrkB. In the presence of DEX, a reduction in Sph2-TrkB interaction (which is required for ERK pathway activation) was observed suppressing the expression of BDNF-induced synaptic proteins in cortical cultures[135].

Another study investigated the acute and chronic effects of CORT on TrkB expression in young and mature neurons derived from primary mouse cortical neurons. Following acute CORT exposure, an increase in TrkB protein levels was observed in early primary cortical neurons but not in mature neurons derived from the same primary cortical cells. Subsequent experiments indicated that this increase may be mediated via c-Cbl, which was shown to co-precipitate with TrkB in the presence of CORT. This CORT-induced increase in TrkB activation was prevented when c-Cbl was knocked down. Following chronic CORT exposure, a significant decrease in TrkB levels was observed in both early and mature cortical neurons. Interestingly, *c-Cbl* mRNA levels have been found to be decreased in both the frontal cortex of mice subjected chronic stress and in the prefrontal cortex of human suicide subjects[136].

Numakawa *et al*. (2009) demonstrated that chronic (24 or 48 hours) exposure to DEX decreased BDNF-mediated release of glutamate via inhibition/suppression of PLC-γ/ Ca^{2+}-signaling in rat cortical neurons. In addition, the interaction between TrkB and the GR was also reduced following both DEX and CORT exposure, and GR expression was decreased. Interestingly, following *in vitro* siRNA silencing of the GR, the inhibitory/ suppression effects of DEX on PLC-γ/Ca^{2+}-signaling were replicated while the opposite was observed following GR overexpression. These results suggest the importance of TrkB-GR interaction in the face of BDNF-induced PLC-γ activation needed for the release of the neurotransmitter glutamate[137].

To investigate whether antidepressants or nutraceuticals (i.e. alternative products derived from herbs and dietary supplements sometimes used for medicinal purposes[138]) can counteract the effects of GCs on cell viability and neuronal plasticity, Gite *et al*. (2019) used SH-SY5Y cultures. They observed a reduction in cell viability following CORT exposure, in addition to a reduction in mRNA expression of cAMP-responsive element binding protein (CREB)1 and BDNF-VI, both mediating neuronal survival and synaptic plasticity[139]. These effects were shown to be reversed following addition of antidepressants and a few selected extracts.

Cellular processes underlying GC effects

As explained above, genetic and epigenetic processes underly molecular mechanisms of GC-induced effects in relation to SRDs. The altered molecular processes can manifest in affected cellular processes too. These may include neurogenesis, synaptic plasticity and neuronal activity, all processes that have been implicated in SRDs. See Table 1(c) for a summary of the listed studies.

Neurogenesis

The formation of new and functional neurons from their precursors is referred to as neurogenesis[140]. Neurogenesis mainly takes place during early development, although the existence of adult neurogenesis has been firmly established in rodents, while still debated in human. Mechanisms underlying neurogenesis have been extensively studied using *in vitro* neuronal models, by looking at proliferation, differentiation, cell death and survival[141].

The process of neurogenesis is influenced by many factors including hormonal exposure. CORT, for example, has been shown to influence the number of proliferating NSCs and their survival[142]. While data on CORT affecting proliferation indicates both increased as well as decreased proliferation, the overall impact of CORT on neurogenesis seems to be a reduction in the number of differentiated and functional new neurons, likely through priming of cells for gliogenesis[143-145].

There is a lack of consensus on the impact of GCs on neuronal physiology. While some studies report a decrease in viability of NSCs (mainly via apoptotic pathways) with increasing concentrations of GCs[146, 147], one study observed no change in cytotoxicity and cell survival of HT22 mouse hippocampal neuronal cultures with even higher concentrations of CORT[148] compared to the aforementioned studies. In contrast, increases in neural progenitor proliferation have also been documented *in vitro* following GR activation. For instance, Anacker *et al*. observed increased proliferation (as shown by Bromodeoxyuridine (BrdU) staining) and astrogliogenesis, and decreased neurogenesis (MAP2-positive and DCX-positive cells) following low CORT concentrations (100 nM) in immortalized human hippocampal progenitors. High concentrations (100 μM) however, led to decreased proliferation and differentiation (replicated in[149] in human hippocampal progenitors). The effects of low CORT concentrations were mediated by the activity of the MR, while the effects of high CORT concentrations seemed to be mediated by GR activity, as demonstrated by co-incubation with receptor antagonists. The underlying molecular pathways which were impacted by CORT exposure involved Notch/Hes-signaling in conditions with low CORT concentrations, and TGFβ-SMAD2/3

signaling with high CORT concentrations[60]. A decrease in proliferation but not differentiation has also been reported in adult rat hippocampal progenitors following a 5μM concentration of DEX[150, 151]. In Yu *et al*. (2004), CORT exposure (2 μM) in fetal hippocampal progenitor cells led to a decrease in both proliferation and differentiation.

MR is highly expressed in the brain, particularly in the hippocampus, and, together with GR, plays a crucial role in neuronal survival[152]. In line with *in vivo* findings*, in vitro* studies have demonstrated that MR activation and overexpression reverses GC-induced hippocampal neuronal apoptosis via the GR[82, 153]. These studies highlight the importance of MR activity in stimulating neuronal survival in the presence of GCs. It is important to mention that these neurotoxic effects are most often seen in the presence of high GC concentrations. That being said, identifying the target receptor of interest (GR, MR, or both), which will inform the selection of non-synthetic or synthetic GCs (e.g., DEX for GR or aldosterone for MR), and eventually GC concentration are crucial parameters in drawing conclusions on the effects of GCs *in vitro* and will be discussed further below.

Downstream transcription factors are required for the synthesis of proteins and growth factors involved in neurogenesis. For example, a study by Tamura *et al*. (2005) looked at changes in mRNA expression of Tolloid-like 1 (*Tll-1*) – a metal-based protease enzyme – whose function is required for the synthesis and functioning of bone morphogenetic proteins (BMPs) required for neurogenesis in the hippocampus of adult mammals. Following exposure to CORT, a decrease in *Tll-1* promoter activity was observed in cultured SH-SY5Y cells. Additionally, this decrease was also associated with a reduction in endogenous mRNA levels of *Tll-1*. Together, these *in vitro* results suggest a role of Tll-1 in modulating neurogenesis *in vivo* in the presence of a stress stimulus[154].

A stress-induced decrease in neurogenesis has been proposed as a possible underlying mechanism for the observed hippocampal atrophy in patients suffering from SRDs such as MDD and PTSD[155, 156]. Antidepressants for instance, have been shown to reverse stress-induced hippocampal volume reduction in both animals and humans[157, 158]. *In vitro,* antidepressants have also been shown to reverse the GC-induced decrease in neurogenesis[159, 160].

The use of stem cell technology allows the investigation of the effects of GC exposures not only on proliferating progenitors but also on post-mitotic neurons *in vitro*. For instance, a decrease in viability was observed in a study using human ESC-derived NSCs and differentiated SH-SY5Y cultures. Higher concentrations (100 μM) of DEX led to a decrease in proliferation (as assessed by BrdU) of hESC-derived NSCs, a decrease in

the percentage of cells bearing neurites, and an increase in apoptosis[161]. Conversely, lower concentrations of DEX (50 nM) induced NSC proliferation and decreased differentiation of human iPSC-derived neurons[143]. Similarly, DEX (50 μM) and CORT (at varying concentrations) also induced proliferation in human iPSC-derived NPCs. Under oxidative stress conditions, CORT alone, but not DEX, promoted proliferation. The authors concluded that these results highlight the importance of MR activation in conferring the neuroprotective effects during cellular stress conditions[144]. This further illustrates the differential effects of the MR when compared to GR, with increased MR activity being associated with protective effects in the brain, whereas decreased activity linked to psychiatric disorders[82].

Prenatal stress and early exposure to chronic stress have been proposed to increase risk of neurodevelopmental disorders in humans[162]. There is evidence of parallel effects of increased prenatal GC signaling and prenatal stress[55], although the exact link might not be straightforward. To better understand the effects of GCs on neuronal development, a recent study exposed human iPSC-derived cerebral organoids to DEX (100 nM) for an acute period of 12 hours and observed a non-cell-type specific expression and activation pattern of *NR3C1*. DEX resulted in an increased transcriptional response of GR-regulated transcripts, such as *FKBP5*, and an accumulation of GR in the nucleus, indicating that DEX activated GR-signaling in cerebral organoids. An increase in *PAX6* in both neural progenitors and neuronal clusters suggests increased proliferation of both progenitor cells and an increase in immature neurons[56]. Many of the differential expressed genes are known to play a crucial role during neuronal development by regulating neuronal proliferation and safeguarding the neural progenitor pools[163-166]. The acute exposure (12 hours) was not sufficient to lead to changes in cell number but was able to prime the cells transcriptionally for altered developmental milestones. Their findings validate previous *in vitro* studies showcasing effects of prolonged GR activation on neurogenesis, and neuronal maturation[113]. Additionally, DEX-induced gene expression changes within neurons alone were shown to be associated with certain brain behavioral phenotypes and risk for psychiatric phenotypes including MDD, neuroticism, openness, sleep-associated behaviors, intellectual disability, and autism spectrum disorder. Thus, this *in vitro* model is a great first step forward and may serve as a proof-of-concept for the use of increasingly complex *in vitro* human cell models such as 3D cerebral organoids (and maybe one day assembloids of hypothalamic, pituitary, and adrenal organoids) in order to enhance our biological understanding of gene-environment interactions. Even though they do not include vasculature and supporting glial cells, they are characterized by a cytoarchitecture and a heterogeneous population of NPCs (which is seldom considered) that highly resembles *in vivo* conditions.

Synaptic plasticity

Following the generation of a new neuron, synapse formation is one of the next crucial steps in neurodevelopment[141]. Synaptic plasticity is a physiological process where defined patterns of neural activity lead to long lasting alterations in synaptic functioning and neural excitability. This basic process underlies fundamental functional abilities of the brain such as information storage, and brings about changes in complex behaviors[167]. Conditions of stress have been shown to impact synaptic plasticity, long-term potentiation (LTP), synaptic potentials, and neuronal activity.

LTP

Impairment of LTP – an increase in synaptic strength – has been observed in adult mice following acute stress[168]. For example, negative effects of CORT on LTP have been shown to be dependent on $GABA_A$ receptor blockage and **β-adrenergic activation**, as seen in an *ex-vivo* study looking at rapid effects of GCs in the hippocampus[169].

GC-induced changes in LTP have also been linked to GR expression in hippocampal dendritic spines. Acute exposure to DEX in hippocampal slices led to an increase in phosphorylated (p)-Cofilin and extracellular signal-regulated kinase (ERK)1/2, which is known to play a role in the regulation and stabilization of cytoskeleton actin filaments in spines. Paradoxically, a reduction of (p)-Cofilin levels in spines was also observed after DEX exposure. Together, these results highlight the role of GR in hippocampal dendritic spine function and in the local effects of DEX on synaptic plasticity, specifically on spine actin remodeling[170].

Neuronal activity

CORT has been shown to cause rapid changes in hippocampal activity, by increasing the rate of miniature excitatory postsynaptic potentials (mEPSPs) which can modulate presynaptic properties, trigger an action potential, and eventually lead to glutamate release[80]. These rapid effects of CORT seem predominantly mediated via the MR and not the GR, causing initial non-genomic changes that are later manifested through genomic signaling pathways. This study highlights MR-GR interplay and indicates a role for MR as a "cortico-sensor" enabling fast non-genomic responses to CORT. Once the MR effects have returned to baseline, it is followed by GR-mediated genomic downstream alterations, illustrating the dual mechanism of CORT leading to both short and long-term changes in hippocampal activity in response to stress.

Well-regulated intracellular Ca^{2+} dynamics are essential for neuronal survival, synaptic plasticity and function[171]. A chronic exposure to GCs leading to increased levels of intracellular Ca^{2+} negatively impacts neuronal survival and plasticity[172, 173]. Therefore,

several studies have investigated the effects of GCs on Ca^{2+} influx in neurons *in vitro*[134, 147, 174, 175]. One study noted that DEX enhances the toxic effects of amyloid β-protein-induced increases in neuronal Ca^{2+} influx. Interestingly, DEX alone had no effect on Ca^{2+} influx in hippocampal neurons following a 24 hour exposure[147]. Bhargava *et al.* (2002) investigated the effects of GCs on Ca^{2+} transients in hippocampal-derived H19-7 neurons and demonstrated that GCs inhibit the plasma membrane protein Ca^{2+} - ATPase-1 (PMCA1) in these hippocampal cultures, which is needed for detecting intracellular Ca^{2+} levels in neurons. Following CORT exposure, an increase in Ca^{2+} transients was observed in hippocampal-derived H19-7 neuronal cultures, independent of calcium channel activation[174]. Another study observed a decrease in basal Ca^{2+}levels in rat cortical neurons following DEX or CORT exposure, or physiological and pathological levels of glutamate[176]. Chen *et al.* (2011), demonstrated a reduction in intracellular Ca^{2+} concentration, via Ca^{2+} pumps following high concentrations of DEX in primary rat hypothalamic neurons[177]. Kumamaru *et al.* (2008) also investigated the effects of DEX on Ca^{2+} influx and observed a decrease of post-synaptic Ca^{2+} influx induced by BDNF[134]. Together, these results highlight the role of GCs in regulating Ca^{2+} levels that are required to ensure proper neuronal functioning, calling for increased studies into this mechanism.

Mitochondrial function

GCs have also been shown to play a role in regulating the functioning of mitochondria, which are responsible for generating energy in cells. The mitochondrion for instance is important in facilitating adaptation to stress. Particularly, GCs can inhibit the activity of enzymes involved in the mitochondrial electron transport chain, and even increase levels of mitochondrial reactive oxygen species (ROS)[178]. Du et al. tested low and high doses of CORT exposure in primary cortical neurons on mitochondrial function. While low concentrations showed neuroprotective effects, higher concentrations led to neurotoxicity through increased levels of kainic acid. The mechanisms of action of high doses of CORT in cortical neurons was shown to include a decrease in the GR/Bcl-2 complex translocation into the mitochondria following acute treatment. Prolonged high CORT treatment however, led to a decrease in GR and Bcl-2 expression[179]. Another study by Luo et al. investigated the effects of CORT on a Bcl-2 associated protein, Bag-1 (Bcl-2 associated athanogene), in GR translocation into the mitochondria. Acute and high concentrations of CORT increased the generation and translocation of the GR/Bag-1 complex into the mitochondria in primary cortical neurons. Bag-1 was demonstrated to regulate GR translocation, with increased expression of Bag-1 inhibiting mitochondrial GR levels following prolonged and high CORT concentrations[180]. Together these results suggest a concentration- and exposure-dependent response of GCs on mitochondrial function, neuronal survival, and GR mitochondrial translocation. This has further impli-

cations for the role of mitochondrial function in conferring resilience or susceptibility to GC challenges in neurons, highlighting that mitochondrial-associated pathways might be potential therapeutic targets for psychiatric disorders[181].

Neurotoxicity

Inflammation and the activation of inflammatory signalling pathways, in part due to increases in circulating cytokines, have been related to stress and SRDs[182]. A range of cellular studies have provided evidence that NF-κB transcription has an effect on several neuronal processes including proliferation, maturation, and neurogenesis in the presence of stress[183]. In one *in vitro* study, a single exposure to DEX before the addition of amyloid β fragment 25-35 increased the vulnerability of hippocampal neurons to the inflammation-inducing effects of amyloid β by increasing intracellular calcium levels, and decreasing nuclear levels of NF-κB[147, 184]. Another study on the effects of GCs on NF-κB expression in hippocampal neurons, reported an increase in protein expression of several NF-κB subunits including p50, p56, p-p65 and A-p65 after exposure to CORT for 48 and 72 hours[184]. These *in vitro* findings indicate that GCs induce an increase in NF-κB transcriptional activity in the hippocampus, which in turn carries anxiogenic properties.

Evidence reflecting oxidative damage has been documented in rodent models of chronic stress. Bharti *et al*. (2018) exposed HT22 mouse hippocampal neurons and primary cortical neurons to CORT and investigated Thioredoxin (Trx), a protein involved in regulating oxidative protein cysteine changes. While no changes in protein levels of Trx and its reduced form were observed following chronic CORT exposure, a substantial increase in the endogenous Trx inhibitor, Txinp, was observed. Interestingly, this was reversed in the presence of the GR antagonist RU486, also known as mifepristone[185].

In another study using SH-SY5Y cell cultures, CORT treatments led to an increase in levels via upregulation of nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase and induced the production of ROS. These effects were reversed in the presence of NAPDH oxidase inhibitors which suggests an underlying mechanism of SRDs, particular MDD, with NAPDG oxidase inhibition being a potential therapeutic target to pursue[186].

Loss of neurons in the CNS is a neuropathological hallmark of neurodegenerative disorders, and can be mediated via inflammatory mechanisms[187]. Elevated levels and recurrent exposure to GCs are known to induce neurotoxicity. Kim *et al*. (2018) acutely exposed human SK-N-SH neuroblastoma cells to high concentrations of CORT. Following CORT exposure, they observed a reduction in cell viability (also reported in another study using SH-SY5Y cells[188]) and in cellular ATP levels linked with an increase

of caspase-3/7 activity – early markers of apoptosis. Mitochondrial function was also impaired via decreased mitochondrial membrane potential, and levels of ROS were increased, including mitochondrial superoxide[189].

Other studies have demonstrated anti-inflammatory effects of DEX in rat embryonic cortical neurons co-cultured with microglia stimulated with interferon-gamma and lipopolysaccharide. DEX exposure was shown to downregulate the expression of nitric oxide and inducible nitric oxide synthase produced by microglia, which are known to be neurotoxic to neurons when present in high levels[190]. That being said, studies into the neurotoxic effects of GCs remain controversial and highly dependent on several conditions such as exposure time, intensity of stimulus amongst others.

GC effects on glial cells

Although the previous section focused on GC effects on neurons, an increasing number of studies are highlighting the roles of glial cells in SRDs and their involvement in GC effects. Glial cells including astrocytes, oligodendrocytes, and microglia play essential roles in the regulation, support, and protection of neurons[191]. A summary of the listed studies can be found in **Table 1**(**d**).

Microglia

Microglia dysregulation has been suggested to be an underlying cause of immune dysregulation seen in MDD patients[192]. Changes in microglia activation, morphology and in the level of activation markers have been reported in post-mortem brain samples of subjects with MDD[193]. One particular *ex vivo* study by Snijders *et al*. investigated responsiveness of microglia taken from post-mortem brain tissue of MDD patients to GCs. Following a 72-hour exposure to DEX, an increase in *CD163* and *MRC1* expression ('anti-inflammatory' response genes) was observed, with no change in microglia activation markers. These results suggest that GC-induced microglia responsiveness is affected in patients with MDD[194, 195].

Astrocytes

Astrocytes play a critical role in regulating the neuronal environment. Recent cell culture studies using primary cortical astrocytes reported that exposure to CORT or DEX was associated with a reduced proliferation of astrocytes which may be mediated via downregulation of GR expression[196, 197], and decreased glucose transport and affinity of glutamate uptake in astrocytes[198].

More recently, MDD patient-derived astrocytes were generated from iPSCs and exposed to CORT. Unique transcriptomic responses were observed following acute (24 hrs) and chronic (7 days) treatment with CORT. Subsequent whole transcriptomic sequencing identified a unique expression profile following chronic CORT in MDD patient-derived astrocytes, with the differentially expressed genes being associated with synaptic signaling, ion homeostasis, and GPCR ligand binding[199]. These studies highlight the unique effects of GCs in astrocytes, specifically in MDD patients, and offer several opportunities for future research looking into the role of astrocytes in inferring risk for SRDs.

Oligodendrocytes

Myelination, a process driven by oligodendrocytes, is vital for the healthy functioning of neurons. In a recent study, the effects of prolonged exposure to both CORT and DEX were investigated on changes in morphology and immunoreactivity of oligodendrocytes and astrocyte-associated proteins[200]. This study reported a dose-dependent decrease in the co-localization between myelin basic protein (MBP) and phosphorylated neurofilament, termed the myelination index, in spinal cord- and cortical myelinating neuronal cultures. This study reported a decrease in immunoreactivity of MBP and of connexin-43 in both rat embryonic spinal cord and cerebral cortex primary cultures (in both glial cultures and glia-neuron co-cultures) after prolonged exposure to GCs. These effects were prevented by the GR antagonist RU486. These results indicate the toxic effects of CORT on myelin formation *in vitro,* partially mediated via the GR.

Together these results highlight the importance of glial cells in conferring susceptibility to GCs *in vitro*. More studies looking into the interplay of glial cells and neurons via co-cultures in response to GC stimulation, will shed light on how both cell types interact to confer GC-induced effects on vital neuronal functioning.

GC effects on neurotransmitter systems

Effects of GCs on neurotransmitter systems are complex and involve multiple levels of regulation, including modulationg of gene expression, protein synthesis, and neurotransmitter system release and uptake. This section will provide an overview on *in vitro* studies investigating effects of GCs on neurotransmitter signaling systems. A summary of the listed studies can be found in **Table 1**(**e**).

Glutamatergic signaling

Changes in glutamate transmission and release have been observed following exposure to GCs *in vivo* and *in vitro*[201, 202]. Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepro-

pionic acid receptor (AMPAR) trafficking is essential for the transmission of fast excitatory synaptic activity in the brain. AMPAR trafficking may modulate synaptic plasticity, where increased membrane recruitment of the receptor leads to synaptic potentiation, and increased receptor endocytosis leads to synaptic depression[203]. Two studies investigated the effects of CORT alone[204] or in combination with the b1- and b2-adrenergic receptor agonist isoproterenol, which facilitates synaptic potentiation[205], on AMPAR activity and trafficking in both *in vitro* primary hippocampal neurons and *ex-vivo* rat coronal brain slices. Short-term CORT (but not isoproterenol) induced increased AMPAR glutamate receptor 2 (GLuR2; a AMPAR subunit) surface mobility and synaptic surface expression exclusively via the activity of MRs, which eventually facilitates potentiation. However, in the long-term, CORT slowly increased surface GluR2 and trafficking, exclusively via the activity of GRs, leading to impeding synaptic potentiation[204]. Hence, this CORT-induced increase in AMPAR surface trafficking is time- and receptor-dependent and carries consequences on the regulation of synaptic plasticity. Zhou *et al*. showed that CORT alone had no effect on AMPAR phosphorylation, surface expression of GluA1 and GluA2 or miniature excitatory postsynaptic currents (mEPSCs). However, increased AMPAR phosphorylation, GluA1 and GluA2 expression, and decreased inter-event interval of mEPSCs was seen when isoproterenol and CORT were combined together[205]. These results highlight the interaction between GC and adrenergic signaling on glutamate transmission.

Besides AMPA-signaling, glutamatergic transmission is also affected by the expression and functioning of the N-methyl-D-aspartate receptor (NMDAR). Mahmoud and Amer (2014) investigated the effect of GC exposure on hippocampal activity by investigating the effects of CORT on the protein expression of NDMAR subunits NR1, NR2B, and NR2A[206]. The protein levels of these subunits were decreased following exposure to low dosages of CORT, which was reversed in the presence of growth hormone (GH), highlighting how GC-induced effects on synaptic transmission are reversed in the presence of low doses of GH. This study highlights that GC-induced effects involve the inhibition of neuronal processes via NMDAR activity[207].

Noradrenergic functioning

The noradrenergic system in the brain is one of the key players and regulators of the stress response together with glucocorticoid signaling. Interestingly, it is implicated in stress-related affective disorders such as MDD and PTSD[208]. Noradrenergic mechanisms, which involve norepinephrine (NE), play a key role in the process of fear conditioning and in the development of PTSD[209]. The mechanisms involved in fear conditioning are suggested to be mediated by the release of NE in the amygdala, strengthening the experience of fear conditioning. In PTSD, the process of fear condi-

tioning is dysregulated, and it has been suggested that the noradrenergic system is overactive, leading to an exaggerated fear response, a key symptom associated with PTSD[210].

To investigate the effects of stress and GCs on noradrenergic functioning, Fan *et al.* (2018) investigated the effects of CORT in SH-SY5Y cells on the expression of *Phox2a* and *Phox2b* – two homeodomain transcription factors – that are crucial in the development of noradrenergic neurons during embryonic development. Increased expression of these two transcription factors was observed following exposure to CORT as a stressor[79]. Moreover, in *ex-vivo* rat brain slices and within the basolateral amygdala, CORT reversed the LTP-inducing effects of isoproterenol. This suggests that GCs can reverse the effects of β-adrenergic signaling[211].

Serotonergic system

Aberrant functioning of the brain serotonergic system has been associated with SRDs like MDD and documented in human[212], animal[213], and *in vitro* studies[214]. Serotonin levels increase following stress, which has a modulatory effect on the functioning of the HPA-axis, limiting the negative consequences of a prolonged activation on inflammation and oxidative stress[215]. Chronic exposure to stress can in contrary lead to decreased levels of serotonin, which is associated with the development of MDD symptoms, namely changes in mood, sleep patterns, and appetite[216]. Antidepressants such as selective serotonin reuptake inhibitors (SSRIs), work on this system by increasing the levels of serotonin in the brain, and as such reversing the symptoms of MDD[217].

Monoamine oxidase (MAO) is an enzyme responsible for the breakdown of neurotransmitters including serotonin, and inhibitors of MAO are widely used as antidepressants[218]. *In vitro*, increased expression of two MAO isoforms, MAO A[213, 219] and MAO B[220], is observed following exposure to DEX. Some studies furthermore demonstrate the inhibitory effects of MAO inhibitors on DEX-induced increased MAO catalytic activity[220], apoptosis and a decrease in cell survival[221].

Conclusion and future considerations

We provide an extensive overview of *in vitro* research findings (**Figure 4**) on the effects of GCs on different types of neuronal cultures. It has become clear that *in vitro* studies aid in unraveling the multiple GC-induced cellular and molecular pathways implicated in SRDs. Advances in stem cell technology opens avenues for the investigation of gene-environment interactions that is fundamental in understanding the pleiotropic

risk to develop SRDs. GC effects differ across central nervous system cell types (i.e., neuronal subtypes) and depending on whether GC treatment is acute or chronic. It is apparent that *in vitro* studies are split into separate categories. While many studies aim to investigate the underlying mechanisms of GCs, others make use of *in vitro* studies as a validation for *in vivo* findings, and a smaller number of studies aim to test the neuroprotective effects of drugs or nutritional supplements on GC-induced toxicity. As the literature shows a great diversity in experimental conditions, it is not surprising that results remain conflicting.

Nevertheless, *in vitro* neuronal models (especially stem cell-based models) are increasingly showing relevance and promise not only in investigating effects of GC exposure which would allow us to unravel mechanisms underlying stress susceptibility and resilience, but also in their validity in translational clinical efforts, such as the identification of biomarkers[113], close to identifying potential novel therapeutic targets. Therefore, tackling the challenges and limitations that come with *in vitro* setups to investigate effects of GCs is instrumental in order to better understand biological processes moderating and/or mediating the onset and course of SRDs. More elaborate systematic reviews or meta-analyses should be conducted including the different conditions and parameters such as exposure time, concentration range, cell line, and age of culture, to provide a more accurate representation of GC effects *in vitro*. Advances in stem cell technology such as 2D and 3D patient-specific generation of neuronal and glial cultures are expected to help gain new knowledge about individual mechanisms contributing to disease that cannot be understood with human or animal studies alone. Therefore, improved standardized GC paradigms *in vitro* that better reflect *in vivo* conditions during stress could provide useful insights to apply in advanced and complex culture models[53]. A few suggested steps to take could include: (1) selecting the appropriate model based on its characteristics (see **Figure 3** for reference) and its potential to answer the research question; (2) selecting the model based on GR/MR expression and model responsiveness to GCs, (3) defining whether acute or chronic exposure is more appropriate, and (4) defining parameters and conditions including concentrations, exposure time, and culture conditions such as the use of culture media in the presence or absence of certain factors with masking effects (e.g., growth factors). For instance, making use of concentrations that are more representative of *in vivo* conditions (as explained in[179]) would aid in establishing the much needed standardization of *in vitro* studies investigating GC-associated mechanisms. Finally, the question of how best to study the effects of two or more stress mediators together (e.g., NE and CORT) is particularly important and highlights another important challenge that needs to be addressed in future studies.

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Chapter 3

Chronic Cortisol Leads to Negative Alterations in Neuronal Processes at Different Stages of Neuronal Development in Human Embryonic Stem Cell-Derived Cortical Neurons

EMBARGO

Publication in preparation:

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Chapter 4

Identification of a Chronic Cortisol-Responsive Transcriptomic Profile Throughout *in vitro* Differentiation of Human Embryonic Stem Cell-Derived Cortical Neurons

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Based on publication under review in Cells:

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Chapter 5

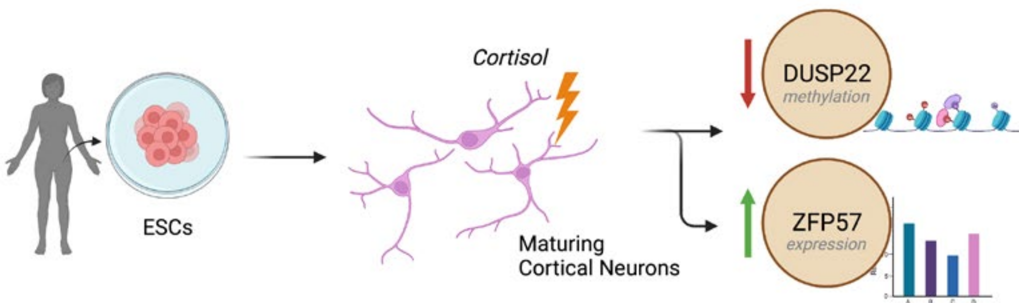
Effects of Chronic Cortisol Effects on DNA Methylation, mRNA and Protein Levels of *DUSP22* and *ZFP57* in Human Cortical Neurons

Bassil K., Riemens, R., Albeirakdar, E., de Nijs, L., Kenis, G., & Rutten, B.P.F.

Abstract

Post-traumatic stress disorder (PTSD) is characterized by exposure to severe psychological stressors with prolonged or chronic impact. While the exact molecular processes that mediate and moderate the impact of these stressors on the clinical expression of PTSD are still unclear, accumulating evidence suggests that epigenetic mechanisms, including DNA methylation, may play a role. Previous epidemiological studies have identified differential changes in DNA methylation in the *DUSP22* and *ZFP57* genes, to be associated with differential susceptibility of developing PTSD in humans exposed to severe stressors. In this pilot study, I aimed to validate these findings in an experimental human neuronal cell model and explore whether DNA methylation and expression levels of these two genes actual mediates the impact of chronic stress on biological processes and physiology. To do so, I investigated DNA methylation and expression profiles of these candidate genes in an *in vitro* model of human embryonic stem cell-derived cortical neurons exposed to chronic cortisol. The results of the pilot study showed that chronic cortisol exposure led to differential DNA hypomethylation in *DUSP22* and increase in *ZFP57* expression levels in maturing cortical neurons *in vitro*. These preliminary findings highlight the need to further investigate the role of these candidate genes *in vitro* and elucidate their putative roles in the molecular underpinnings of PTSD susceptibility.

Keywords: stress susceptibility, cortisol, PTSD, epigenetics, DNA methylation



(Figure created with BioRender.com)

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating mental health disorder that can develop in response to a traumatic experience[1]. Symptoms of PTSD include intrusive memories and thoughts, avoidance behavior, negative mood, and hyperarousal. Early life stress in the form of childhood trauma increases the risk of PTSD later in life[2]. One of the possible mechanisms mediating the negative effects of traumatic stress could be due to elevated and chronic exposure to cortisol (CORT). Stress-related disorders (SRDs) including PTSD have been associated with biological changes in CORT signaling[3, 4]. Trauma recollection has been linked to increased CORT responsiveness[5], and dysregulation in the hypothalamic-pituitary-adrenal (HPA) axis has been previously associated with PTSD patients[6, 7]. Despite advances in our understanding of the pathophysiology of PTSD, the molecular mechanisms involved remain elusive. Even so, it is still not understood why some individuals develop PTSD after trauma, while others retain mental health[8].

There is accumulating evidence of the involvement of epigenetic mechanisms in mediating the effects of environmental stressors[9]. Glucocorticoids (GCs), including CORT, carry genomic-mediated effects through the activation of GC receptors[10, 11], that can alter the expression of key regulatory genes involved in the stress response[12]. In addition to genomic effects, GC receptor activation can also lead to alterations in DNA methylation[13, 14], which in turn carries implications for future transcriptional regulation of downstream genes[15].

PTSD susceptibility has been associated with DNA methylation changes in particular genes[16]. In a recent human study investigating genome-wide DNA methylation changes, longitudinal differences in DNA methylation have been reported in dual-specificity phosphatase 22 (*DUSP22)* and zinc finger protein 57 (*ZFP57)*, among other candidate genes, as being associated to PTSD susceptibility[17]. Therefore, investigating the effects of chronic CORT exposure on gene expression and epigenetic mechanisms in the context of PTSD is important in gaining an improved understanding of molecular and biological mechanisms driving stress susceptibility, ultimately identifying novel biomarkers for prevention or even early treatment of PTSD cases[18].

In this pilot study, I started to investigate DNA methylation and expression profiles of *DUSP22* and *ZFP57* in our previously established *in vitro* model of human embryonic stem cell (hESC)-derived cortical neurons exposed to chronic CORT. We focused on maturing cortical neurons to investigate CORT-induced effects in a post-differentiation cell stage. The objective of this study was to sketch potential interactions between CORT and these

candidate genes, to pave the way for future *in vitro* research investigating their role as key regulators in PTSD susceptibility. Knowledge about the molecular processes that create vulnerability to severe stressful experiences would help in the identification of therapeutic leads and potential biomarkers, both highly needed to advance diagnosis and treatment of PTSD.

Methods

hESC differentiation into cortical neurons

H9 hESC from WiCell were expanded showing a normal karyotype, and expression of pluripotent markers was verified as previously described[19]. hESC were cultured on Geltrex-coated plastic plates (Thermo Fisher Scientific) using E8 flex medium (Thermo Fisher Scientific). Cortical neurons were generated as previously described[19]. In summary, floating embryoid bodies (EBs) were generated by mechanical and enzymatic dissociation of hESC colonies using Gentle (Stem Cell Technologies) and the cells were transferred into AggreWell™800 microwell cultured plates (Stem Cell Technologies) in SMADi neural induction serum-free media (Stem Cell Technologies). EBs were maintained in this media supplemented with a cocktail of SMAD inhibitors for 5 days. Afterwards, EBs were transferred to polyornithine and laminin-coated plates in SMADi neural induction media using a 0.2 µm filter (Corning). Rosette-forming EBs were selected with an enzyme-free neural rosette selection reagent (Stem Cell Technologies) and plated on polyornithine laminin-coated plates to generate neural progenitor cells (NPCs). NPCs were cultured at high density as monolayers and were seeded at lower densities for neuronal differentiation for 1 to 4 weeks. For neuronal differentiation, NPCs were cultured with neural differentiation media supplemented with brain-derived neurotrophic factor (20ng/mL, Peprotech), glial-cell derived neurotrophic factor (20ng/mL, Peptrotech), dibutyryl-cyclic AMP (1mM, Sigma), ascorbic acid (200 nM, Sigma), Laminin (1µg/mL), in BrainPhys™ neuronal media (including N2-supplement A and SM1, StemCell Technologies). The medium was changed every other day for up to 4 weeks. All cell lines were frequently controlled for negative mycoplasma contamination.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min at 4°C. Blocking and cell permeabilization were performed in Tris-buffered saline (TBS) containing 3% donkey serum and 0.1% Triton X-100 for 30 min at room temperature. Primary antibodies, including rabbit anti-DUSP22 (1:500, LS Bio), mouse anti-ZFP57 (1:400, Thermo Fisher), and chicken anti-MAP2 (1:1000, Neuromics), were incubated in the blocking solution overnight at 4°C. Cells were then washed 3 times with TBS for 10 min each. After the washes,

fluorophore-coupled secondary antibodies including donkey anti-mouse or anti-rabbit Alexa Fluor-488 (1:250; Invitrogen Thermo Fisher Scientific), donkey anti-chicken Alexa Fluor-647 (1:250; Invitrogen Thermo Fisher Scientific) dissolved in blocking solution were incubated for 1 h at room temperature. Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) followed by washes with TBS. Finally, slides were cover slipped using 80% glycerol (Merck).

Microscopy

Images were taken using a bright-field Olympus inverted microscope at 10x and 20x magnifications. Fluorescent images were taken using an inverted Stereo Investigator Confocal Spinning Disk (SI-SD) system (MBF Bioscience) at 10x, or 20x magnification.

Cortisol exposure paradigm

CORT (Sigma Aldrich) was reconstituted in DMSO to a stock concentration of 100mM and kept at -20°C. Cells were treated with 100 µM CORT dissolved in 0.001% DMSO or 0.001% DMSO (vehicle control). Maturing neurons were cultured between 4 and 6 weeks and treated during the last week of differentiation for 7 days. CORT or DMSO were renewed every 48 h with every medium change during the treatment period. The cells were collected 24 h after the last renewal of CORT or DMSO containing medium.

RNA extraction

Total RNA was isolated using TriZol according to the manufacturer's instruction (ThermoFisher). Briefly, cells were collected in 1mL Trizol per well of a 6-well plate. Chloroform (200µL) was added to the TriZol samples, inverted a few times, and incubated on ice for 5 min before being centrifuged at 12,000*xg* for 15 min at 4°C. The clear upper phase was transferred to new tubes containing 500µL isopropanol and incubated for 10 min at room temperature to precipitate the RNA. To collect the RNA pellet, samples were centrifuged at 12,000*xg* for 10 min at 4°C. The pellet was rinsed with 75% EtOH, followed by 100% EtOH, while being centrifuged at 7,500*xg* for 5 min after each step. The RNA pellet was dried at room temperature for 30 min and resuspended in 10-20µL of nuclease-free water. RNA was quantified using a Nanodrop™ ND-1000 spectrophotometer (Isogen Life Science).

Real-time quantitative PCR

One µg of total RNA per sample was reversed transcribed to cDNA using a RevertAid H Minust First Strand cDNA Synthesis kit (ThermoFisher) as described in the manufacturer's manual instructions. qPCR reactions were run on the Light-Cycler® 480 system (Roche) using FastStart Universal SYBR Green Master (Rox) mix (Roche) and 700 nM of each primer (Sigma) (Supplementary Table 1), in triplicate per sample. The -2 delta delta

Ct (-2ΔΔCt) method from Livak and Schmittgen was used for calculating the fold gene expression change[20, 21].

Western Blot analysis

Cells from one well of a 6-well plate (around 400,000 cells) were lysed using 1mL 1x RIPA buffer (Thermo Fisher) supplemented with protease and phosphatase inhibitors (Sigma) and incubated on ice for 5 min. Lysed cells were centrifuged at 15,000*xg* for 15 min at 4°C. Protein quantification was performed using the DC™ Protein Assay kit (BioRad). Proteins were separated using 10% SDS-PAGE followed by transfer on a nitrocellulose membrane using the semi-dry blot transfer system (Biorad). Blots were incubated for 1 hour with Odyssey blocking buffer (Licor) then incubated overnight at 4°C with primary antibodies including rabbit anti-DUSP22 (1:500, LS Bio); mouse anti-ZFP57 (1:400, ThermoFisher); and mouse anti-GAPDH (1:2,000,000, Fitzgerald), diluted in Odyssey blocking buffer. The next day, blots were washed once with phosphate buffered saline (PBS) containing 0,1% Tween 20 (PBS-T) and twice with PBS and subsequently incubated for 1 h at room temperature with secondary antibodies (1:10,000; Li-Cor), including goat anti-rabbit IRDye 800 and donkey anti-mouse IRDye 680. Membranes were subsequently washed x3 times with PBS-T and the last wash step was incubated overnight at 4°C. Membranes were scanned and visualized using the Odyssey CLx Infrared Imaging System (Li-Cor, Lincoln). Protein band quantifications were performed using ImageJ (https://imagej.nih.gov), and raw intensity measures were normalized to GAPDH for loading differences.

Pyrosequencing

DNA was extracted and bisulfite (BS) converted using the EZ DNA Methylation-Direct kit (D5020, Zymo Research) following the manufacturer's instructions. From the eluted BS-converted DNA, 1µL was used for the polymerase chain reaction (PCR) amplification followed by BS pyrosequencing for the detection of 5-methylcytosine (5-mC) levels. All BS conversion assays included a negative control.

DUSP22 PCR and pyrosequencing primers (reverse direction) were designed using the PyroMark Assay Design version 2.0.1.15 (QIAGEN) (Supplementary table 2) using Ensembl Genome Browser GRCh37 assemble database. The primers were designed to include the following CpG within DUSP22, with the Illumina probe ID: *cg11235426* (CpG #2). The sequenced region (Supplementary Table 2) included an additional 6 CpG sites that are sequenced (Supplementary Figure 1).

All PCR reactions of BS-converted DNA were performed with FastStart™ Taq DNA Polymerase, dNTP (Roche) following manufacturer's instructions. All PCR reactions had two negative controls (BS negative and water). The melting temperature used in the PCR

was 56°C. Each PCR product (~150bp) was then visualized on a 2% agarose gel in Tris-Acetate-EDTA (TAE) buffer.

Pyrosequencing was performed and quantified using the PyroMark Q48 Advanced Reagents and Pyro Q48 Autoprep 2.4.2 software (QIAGEN) following the manufacturer's instructions. The sensitivity of the assay was assessed using fully methylated and unmethylated DNA standards from the EpiTect PCR Control DNA set (QIAGEN). All the pyrosequencing runs included a negative control and samples were analyzed only after passing quality control.

For the calculation of the global percent methylation of DUSP22, individual DNA methylation percentages of all CpG sites were averaged per condition.

Data analysis and statistics

Each experiment was performed with three independent replicates (unless otherwise stated) per condition for further statistical analysis. Statistical comparisons between conditions were conducted by comparing average values from each condition.

All statistical analysis for cellular and molecular assays were performed with GraphPad Prism 9 (GraphPad, La Jolla). Student's t-test was used to compare means of two independent treatment groups. P-values<0.05 were considered as significant. Data are presented as mean±SEM.

Results

Chronic CORT treatment leads to changes in DNA methylation but not expression levels of DUSP22 in maturing human cortical neurons

We first checked by immunofluorescent staining whether DUSP22 was expressed in our *in vitro* model of hESC-derived cortical neurons. We mostly observed nuclear localization of DUSP22 in MAP2+ neurons (Supplementary Figure 2).

To investigate the effects of chronic CORT on the expression of the transcript and protein levels of DUSP22, I performed RT-qPCR and western blot on maturing cortical neurons treated with CORT or vehicle control. We observed no significant changes in the expression of *DUSP22*, both at the mRNA (p>0.05) (Fig. 1a) and protein levels (Fig. 1b) following chronic CORT.

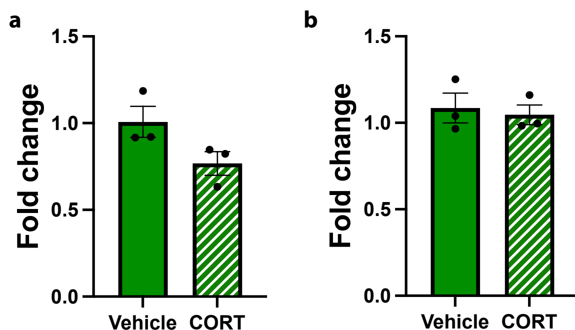


Figure 1. Chronic CORT effects on expression levels of DUSP22 in maturing cortical neurons. **a** Expression of *DUSP22* mRNA expression in CORT-treated compared to vehicle-treated neurons. RT-qPCR results are expressed as fold change. **b** Western blot analysis of the expression of DUSP22 protein in CORT-treated neurons compared to vehicle-treated. Data represented as mean±SEM; n=3; *: p-value<0.05.

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Additionally, with DUSP22 previously reported to be differentially methylated in individuals susceptible to PTSD[17], I investigated using pyrosequencing whether chronic CORT treatment would affect DNA methylation levels of the same genomic region of DUSP22, in maturing cortical neurons. The global percentage of methylation of all the CpGs analyzed within *DUSP22* was not different between chronic CORT and vehicle control (p>0.05) (Figure 2a). Interestingly, when looking at individual CpGs, I showed that CpG #4 was differentially methylated, with a decrease in percent methylation following chronic CORT exposure compared to vehicle (p=0.0324) (Figure 2b). In contrast, CpG #2 that has been reported to be differentially methylated between PTSD susceptible and resilient individuals, did not show any significant methylation differences following chronic CORT treatment (p>0.05) (Figure 2b).

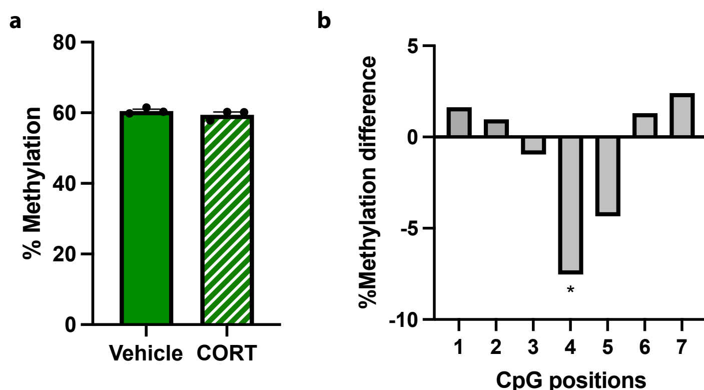

Figure 2. Chronic CORT effects on DNA methylation levels of *DUSP22* in maturing cortical neurons. **a** Percent of global DNA methylation at all CpG site analyzed within *DUSP22* between vehicle-treated and CORT-treated conditions. **b** Differences in percent of DNA methylation at unique CpG sites within *DUSP22* following chronic CORT-treatment over vehicle. Data represented as mean±SEM; n=3; *: p-value<0.05.

Chronic CORT treatment leads to changes in expression levels of ZFP57 and its targets in maturing human cortical neurons

Similar to what was performed for DUSP22, I first started with investigating the expression of ZFP57 by immunofluorescent staining in our *in vitro* model of hESC-derived cortical neurons. We mostly observed nuclear localization of ZFP57 in MAP2+ neurons (Supplementary Figure 3).

Next, I investigated chronic CORT effects on the expression levels of ZFP57. At the mRNA level, I observed a significant increase in *ZFP57* expression after chronic CORT exposure (p=0.0076) (Figure 3a), which was not observed at the protein level (p>0.05) (Figure 3b).

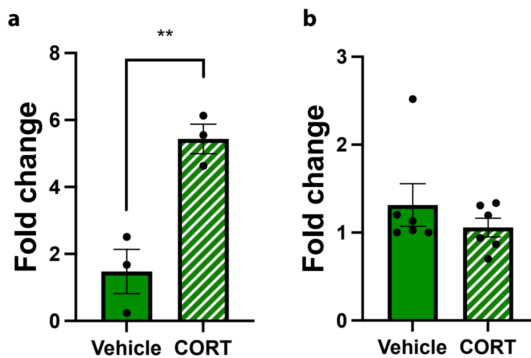

Figure 3. Chronic CORT effects on expression levels of ZFP57 in maturing cortical neurons. **a** Expression of *ZFP57* mRNA expression in CORT-treated compared to vehicle-treated neurons. RT-qPCR results are expressed as fold change. n=3 **b** Western blot analysis of ZFP57 protein expression in CORT-treated compared to vehicle-treated neurons. Data represented as mean±SEM; n=6; **: p-value<0.01.

To better understand the role of *ZFP57* as a transcription factor and mediator of epigenetic mechanisms, in response to chronic CORT, I next looked at the effects of chronic CORT on the mRNA expression levels of direct targets of *ZFP57*, including *DNMT1* and *DNMT3a* isoforms, and *DNMT3b* as a non-direct target. We showed a trend towards decreased mRNA expression of *DNMT1* (Fig. 4a), *DNMT3a(2)* (Fig. 4c), and *DNMT3b* (Fig. 4d), after CORT exposure, however not significant (p>0.05) (Fig. 4).

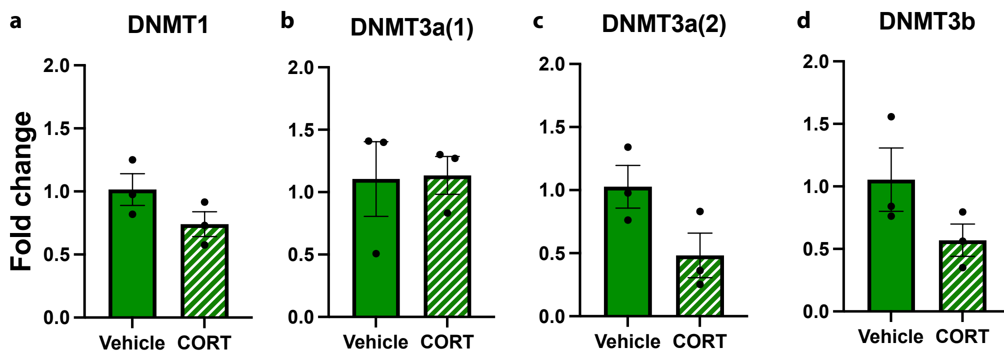

Figure 4. Chronic CORT effects on mRNA expression of *ZFP57* target genes. Fold change expression of **a** *DNMT1*, **b** *DNMT3a(1)*, **c** *DNMT3a(2)*, and **d** *DNMT3b* in maturing neurons treated with CORT compared to vehicle. RT-qPCR results expressed as fold change of CORT in relation to vehicle. Data represented as mean±SEM; n=3; *: p-value<0.05.

Discussion

In this study, I aimed to investigate the effects of chronic CORT exposure on two previously identified candidate genes implicated in PTSD susceptibility, *DUSP22* and *ZFP57*[17]. We first explored the effects of chronic CORT on the expression and methylation levels of DUSP22, as well as the expression levels of ZFP57 and its direct targets, as a potential way to investigate candidate genes *in vitro*. This study was designed as an exploratory investigation into the effects of chronic CORT exposure on candidate genes implicated in PTSD susceptibility.

Firstly, I investigated the potential effects of chronic CORT exposure on DUSP22 expression and methylation levels. While I did not observe CORT-induced changes in DNA methylation within CpG #2, as has been previously demonstrated in PTSD susceptible individuals[17], I showed decreased DNA methylation in CpG #4 located near CpG #2[17] following CORT treatment. DUSP22 is a member of the dual-specificity phosphatase (DUSP) family of proteins[22], dephosphorylating both tyrosine and threonine residues on its substrate proteins. *DUSP22* is expressed in the brain, where it plays an important role in modulating several neuronal signaling pathways[23]. Studies have shown that the atypical DUSP family members (to which DUSP22 belongs) are involved in regulating the activity of several kinases such as MAPK, and play a role in neuronal apoptosis and cell proliferation[22]. The observed differential methylation in *DUSP22* suggests changes to the expression of DUSP22. Despite changes in DNA methylation levels, I did not observe any alterations in DUSP22 expression levels following chronic CORT, similar to

previous findings[25]. This can be explained by evidence suggesting that not all DNA methylation leads to changes in gene expression, as there are various molecular mechanisms including post-translational modifications, that can counteract the effects of DNA methylation. Furthermore, it is possible that the transcriptional regulation of *DUSP22* might be independent of DNA methylation and expression.

Environmental stressors and GCs are known to affect epigenetic mechanisms, such as the regulation of DNMTs in the brain or the activation of GC signaling, followed by downstream genomic-mediated effects[26]. For instance, differential methylation of *DUSP22* has been reported in relation to extreme stress conditions and schizophrenia both *in vivo* and *in vitro*[25]. While this study showed hypermethylation of *DUSP22* following stress conditions, the recurrent association between stressors and DNA methylation of *DUSP22* highlight its potential role in stress susceptibility mechanisms. Moreover, changes in methylation levels of *DUSP22* have been reported not only in PTSD and schizophrenia, but in neurodegenerative disorders as well[17, 24, 25] further highlighting its relevance in brain-associated disorders. Additional research into the effects of chronic CORT on the molecular mechanisms of *DUSP22* methylation and expression is needed to fully understand its role in PTSD susceptibility.

Next, I examined the likely effects of chronic CORT on ZFP57 expression levels and its direct target genes. We observed a significant increase in mRNA expression of *ZFP57,* no change in protein expression, and a trend towards a decrease in the mRNA expression of *DNMT1*, *DNMT3a(2)*, and *DNMT3b*. ZFP57, is a transcription factor of the zinc finger protein family, with little expression in the human brain. It is primarily involved in genomic imprinting and in the maintenance of DNA methylation patterns. It has been shown to interact with DNA methyltransferases (DNMTs), particularly DNMT1 and DNMT3a, which are responsible for DNA methylation[27]. Aberrant expression of *ZFP57* has been linked to abnormal methylation patterns, and changes in ZFP57 methylation levels have been associated with PTSD susceptibility[17] (hypomethylation) and response to PTSD treatment[28] (hypermethylation). The increased mRNA expression of *ZFP57* suggests increased levels of *DNMTs*, although not observed in our study. While this is possibly due to small sample size, another reason could be attributed to the reversing effects of chronic CORT on DNMT expression, which has been shown to decrease following stress and GC exposure[29, 30]. Moreover, changes in DNMT levels have been associated with effects of chronic stress and neurodevelopmental disorders, including schizophrenia[31], and PTSD[32].

While the exact role of ZFP57 has not be fully elucidated in neurons, recent evidence points towards the involvement of ZFP57 in neural differentiation[33]. *ZFP57* knockout ESCs showed decreased expression of the neuronal-specific late marker *TUBB3* as compared to wild-type cultures undergoing corticogenesis, and with dysregulated differentially expressed genes being enriched for neurogenesis and neuronal development processes. While this and similar studies investigating the effects of ZFP57 inactivation can be considered as novel evidence associating *ZFP57* with neuronal processes, the manipulation of ZFP57 expression levels has been performed in early embryonic cell stages, and hence the direct role of ZFP57 in neural differentiation and maturation remains unexplored. With the observed CORT-induced increase in *ZFP57* expression, future research could, for instance, on investigating the effects of chronic CORT on *ZFP57* DNA methylation levels, downstream effects on overall methylation levels, and alteration to neuronal differentiation and maturation mechanisms. Together, the increasing evidence on the involvement of DUSP22 and ZFP57 in neuronal processes and SRDs, makes them interesting candidate genes to further investigate.

Our data highlight that *DUPS22* and *ZPF57* genes are responsive to CORT and may be implicated in the underlying mechanisms of stress susceptibility through alterations in *DUSP22* DNA methylation and gene expression. These preliminary findings should be interpreted with caution and considered as preliminary data that require further validation. Thus, larger and more rigorous studies using similar approaches are needed to further investigate the role of these candidate genes in response to chronic CORT, as well as through loss-of-function and overexpression studies, to gain a better understanding of their effects on neuronal processes and function and their potential contribution to stress susceptibility mechanisms.

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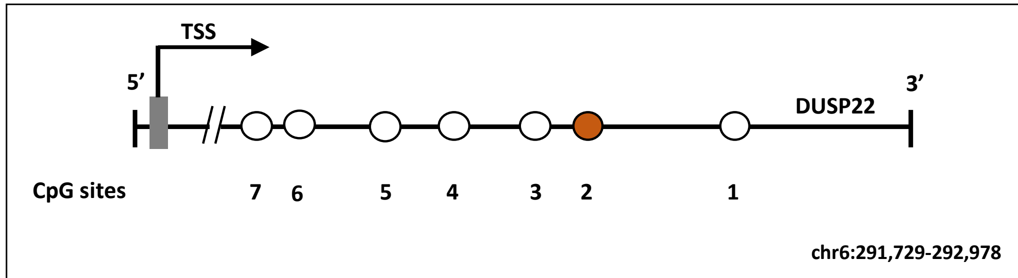
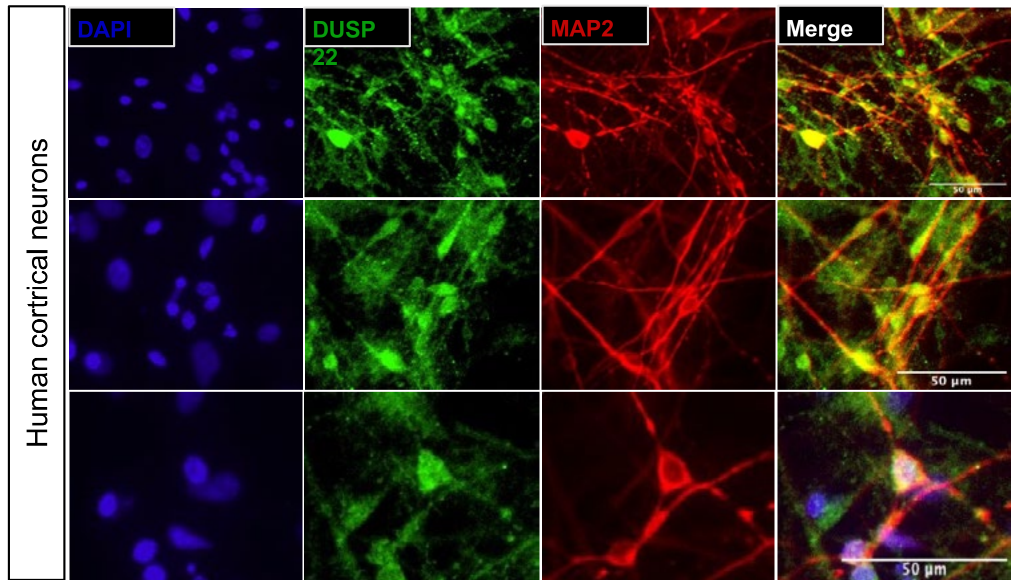
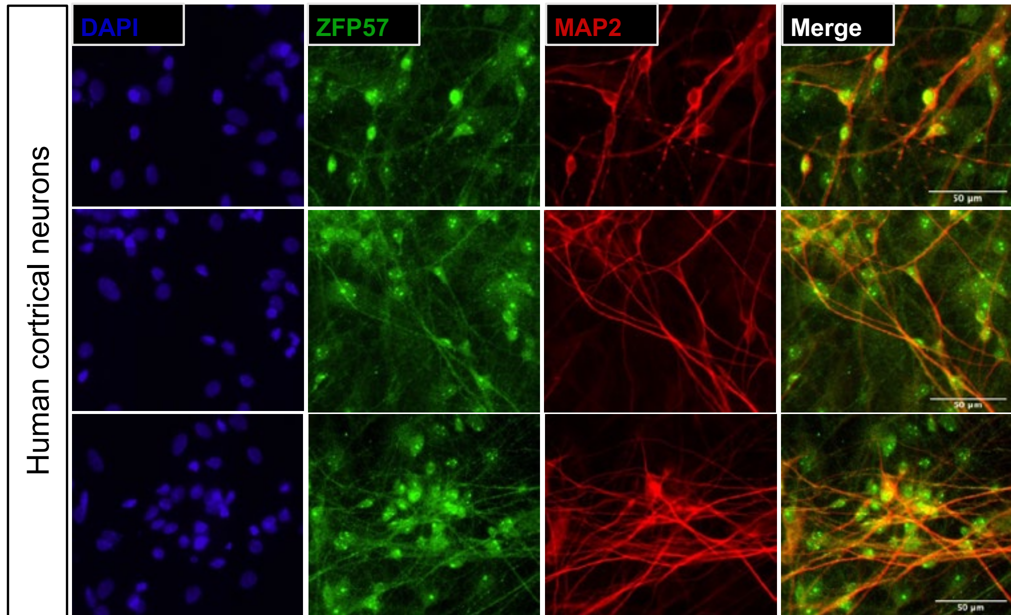
Supplementary information



Supplementary Figure 1. Schematic representation of the analyzed CpGs from the *DUSP22* promoter. CpGs interrogated by pyrosequencing are represented by CpG site numbers according to their relative position. The orange circle pointing to CpG #2 refers to the Illumina probe ID *cg11235426*. The other CpG sites are situated in reference to CpG#2 and numbered accordingly. Circles represent CpG dinucleotides.



Supplementary Figure 2. Representative fluorescence images of human cortical maturing neurons expressing MAP2 (red) and DUSP22 (green), with DAPI-counterstained nuclei (blue).



Supplementary Figure 3. Representative fluorescence images of human cortical maturing neurons expressing MAP2 (red) and ZFP57 (green), with DAPI-counterstained nuclei (blue).

Supplementary Table 1. Primer sequences

Gene	Forward Primer 5′ to 3′	Reverse Primer 5′ to 3′
DUSP22	GGATGACCAAATGACCCTACT	CAGGAGCAAAACACAGCA
ZFP57	Purchased from Genecopoeia.	
DNMT3a(1)	AGAAGCGGGCAAAGAACAGA	CGGGAGCCCTCCATTTTCAT
DNMT3a(2)	AGCGGGTTGTGAGAAGGAAT	CGTCTTTCAGGCTACGATCC
DNMT1	TGGCTTTGATGGAGGTGAAA	CTCCTGCATCAGCCCAAATA
DNMT3b	GATGAAGATCAGAGCCGAGAAC	TCAAAGAGAGGGTGGAAGGA
GAPDH	TTGGTATCGTGGAAGGACTC	CCATCACGCCACAGTTT

Supplementary Table 2. *DUSP22* PCR and sequencing primer overview

Gene	Forward Primer 5′ to 3′	Reverse Primer 5′ to 3′	Target region (GRCh37)	Product size (bp)
DUSP22	GAGGGAAGAAGTTA TTTTGTTTATTTTA	(Bio-)CTCCTCCTCCC TATAACATAC	6:291,729:292,978:1	111

Gene	Sequencing Primer	CpGs	Target region (GRCh37)	PyroMark Orientation
DUSP22	ATTTTATTTTTTATGGTGGTTGA	7	6:291,729:292,978:1	Lower strand (5′-3′)

Abbreviations: Bio: biotinylation; GRCh37: Ensembl GRCh37; bp: base pairs.

Chapter 6

In vitro Modeling of Glucocorticoid Mechanisms in Stress-Related Mental Disorders: Current Challenges and Future Perspectives

Based on publication:

Bassil, K., de Nijs, L., Rutten, B.P.F., Van Den Hove, D.L., & Kenis, G. (2022) In vitro modeling of glucocorticoid mechanisms in stress-related mental disorders: Current challenges and future perspectives. Frontiers in Cell and Developmental Biology, 10.

Abstract

In the last decade, in vitro models has been attracting a great deal of attention for the investigation of a number of mechanisms underlying neurological and mental disorders, including stress-related disorders, for which human brain material has rarely been available. Neuronal cultures have been extensively used to investigate the neurobiological effects of stress hormones, in particular glucocorticoids. Despite great advancements in this area, several challenges and limitations of studies attempting to model and investigate stress-related mechanisms in vitro exist. Such experiments often come along with non- standardized definitions stress paradigms in vitro, variations in cell models and cell types investigated, protocols with differing glucocorticoid concentrations and exposure times, and variability in the assessment of glucocorticoid-induced phenotypes, among others. Hence, drawing consensus conclusions from in-vitro stress studies is challenging. Addressing these limitations and aligning methodological aspects will be the first step towards an improved and standardized way of conducting in vitro studies into stress-related disorders, and is indispensable to reach the full potential of in vitro neuronal models. Here, we consider the most important challenges that need to be overcome and provide initial guidelines to achieve improved use of in vitro neuronal models for investigating mechanisms underlying the development of stress-related mental disorders.

Keywords: stress, glucocorticoids, neurons, *in vitro*, neuropsychiatry

Introduction

Modeling stress and its effects has long been conducted in animal models, with different stress models highlighting different stress mechanisms and processes (e.g., resilience versus susceptibility)[1, 2]. *In vitro* models for stress-related mental disorders (SRMDs), allow the investigation of the effects of key stress hormones (namely glucocorticoids [GCs], norepinephrine, etc.) – independently or in combination - on cellular (e.g., neurogenesis[4]), molecular, and (electro)physiological processes hypothesized to be involved in SRMDs, and more recently on regulation of disorder-specific genetic variants (e.g., *FKBP5[5]*). Additionally, *in vitro* models are relatively cost and time-efficient, and overcome many of the ethical considerations associated with using research animals[6], especially with the discovery of cellular programming and reprogramming technology (CPART) – namely the generation of induced pluripotent stem cells (iPSCs) from adult human somatic cells[3]. Investigating stress mechanisms *in vitro* (as most molecular biology assays) is a highly reductionist approach[7] to understanding stress, its underlying processes, and the mechanisms of SRMDs more broadly. That being said, *in vitro* stress models aim to investigate underlying mechanisms involved in the stress response, as a reaction to exposure to particular stress hormones, with the most studied hormone being GCs[8-19]. In essence, one would assume that investigating effects of GCs *in vitro* seems straightforward. However, the literature shows that GC-induced responses in cultured cells are influenced by many factors, which severely impedes drawing clear, unequivocal conclusions. We believe that increasing the level of standardization in these studies is essential to ensure reproducibility and increased validity of *in vitro* models. It should be acknowledged however, that every experimental setup and design is in fact research question-dependent and as such may require different approaches and conditions. In this perspective, we highlight some of the challenges in investigating the effects of stress hormones in different *in vitro* models by using GCs as an example (**Figure 1**), and formulate recommendations for improvement.

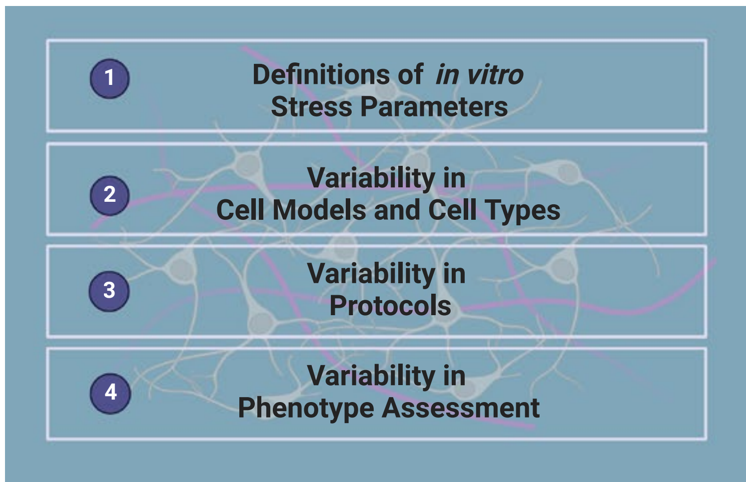


Figure 1. Challenges in investigating the neurobiological effects of GCs *in vitro*. (Figure created with BioRender.com).

State of the art

Modeling aspects of stress in vitro

The neurobiology of stress encompasses a number of mechanisms, including the activation of the autonomic nervous system and the hypothalamic-pituitary adrenal (HPA) axis, with each involving different hormones and regulators such as (nor)adrenaline, corticotropin-releasing hormone (CRH), andrenocorticotropic hormone and GCs[20] (**Figure 2**). Together these mechanisms work in concert to enable an individual to respond to stressors (of an acute or chronic nature) and bring the systems back to homeostasis[2]. Dysregulation of the HPA axis, more specifically an impairment in its negative feedback regulation, has been involved in a number of SRMDs including major depressive disorder (MDD) and post-traumatic stress disorder (PTSD)[21]. Loss of negative feedback leads to HPA-axis hyperactivity in MDD, while the reverse is observed in PTSD resulting in hyporesponsivity of the HPA axis[21]. Prolonged exposure to GCs as a consequence of chronic or repeated stress experiences, has neurotoxic effects which induce several metabolic and cellular vulnerabilities, and which are believed to underlie causative factors in the onset and development of SRMDs[22-25].

GCs such as cortisol (in humans) and corticosterone (in rodents) mediate their effects via two receptors: the glucocorticoid receptors (GR) and the mineralocorticoid receptors (MRs), with the MR showing higher affinity for GCs than the GR[26]. An imbalance in

GR- and MR- mediated responses are thought to increase risk for SRMDs[27]. While MR dysregulation has also been reported in SRMDs[28], the detrimental effects of GCs are predominantly ascribed to GR-mediated signaling, and hence most *in vitro* GC studies focus on the downstream effects of GR activation. This has improved our knowledge on the effects of GCs on several neuronal processes including neurogenesis, neuronal morphology, synaptogenesis, and synaptic plasticity, among others[29], and have helped us better understand the involvement of GCs in SRMDs[30-32].

Recent developments in the field of CPART make the use of *in vitro* stress models even more relevant[3], since it allows to investigate GC-induced cellular responses in the context of the genetic background of individuals expressing differential susceptibility to develop SRMDs. Indeed, the identification of unique gene expression signatures and related pathways implicated in stress vulnerability, have been identified in neurons and glia from iPSCs derived from SRMD patients[18, 32-35]. These models can also be used to examine the effects of genetic risk variants of SRMDs, e.g., polymorphisms in *NR3C1* (the gene coding for GR)[36], or differential responses to GC exposure between iPSC-derived neurons from healthy and SRMD patients[18, 32]. The investigation of the neurobiological effects (at the molecular, cellular, morphological, and physiological levels) of hormones (including cortisol), drugs (incl. antidepressants), or other molecules of interest separately or in combination with one another (e.g., cortisol and (nor)epinephrine; or cortisol and antidepressants) can be performed *in vitro* in a highly controlled environment, without the interference of other systems and molecules. The effects of drugs, hormones, and other molecules on a certain type of neuron implicated in SRMDs (e.g. serotoninergic neurons in MDD; cortical neurons in PTSD), can also be investigated through CPART[3]. Moreover, this technology allows the investigation of pathways and connections between two distinct types of cells (e.g., between different types of neurons or between neurons and glia) in the form of co-cultures (for an example, see[37]). CPART has also enabled the study of the effects of drugs, hormones, or molecules in human cerebral organoids[13] – 3D *in vitro* models of neuronal development, with distinct cellular responses in different types of neural progenitor cells, and neurons. Furthermore, the *in vitro* manipulation of genetic variants (e.g. single nucleotide polymorphisms) or epigenetic mechanisms (DNA methylation of key genes in the stress response, such as *GR* and *FKBP5*) using, for example, recent cutting-edge technology such as CRISPR-cas9, is highly desirable and in many cases more efficient. Finally, *in vitro* studies can be used to model aspects of hypo- or hyper-suppression of the HPA axis, by for instance the manipulation of the GR receptors and sensitivity using agonists and antagonists, in the presence of GCs at different concentrations.

Provided that more reliable and standardized protocols for investigating aspects of stress *in vitro* exist, this may bring about major advances in the areas of stress suscepti-

bility and resilience. *In vitro* modeling could serve as a tool to investigate potential drugs for SRMDs prior to testing on patients, and identify novel target mechanisms, candidate genes, and neuronal subtypes involved. In addition, *in vitro* models may be pivotal as a personalized medicine approach (among others) for SRMD patients[3]. To harness the full potential of *in vitro* models, more complex experimental designs may need to be introduced, such as going from examining the neurobiological effect of only one hormone to a combination of stress mediators, and in defined temporal sequences. Obviously, some degree of standardization in this respect would help in moving the field forward.

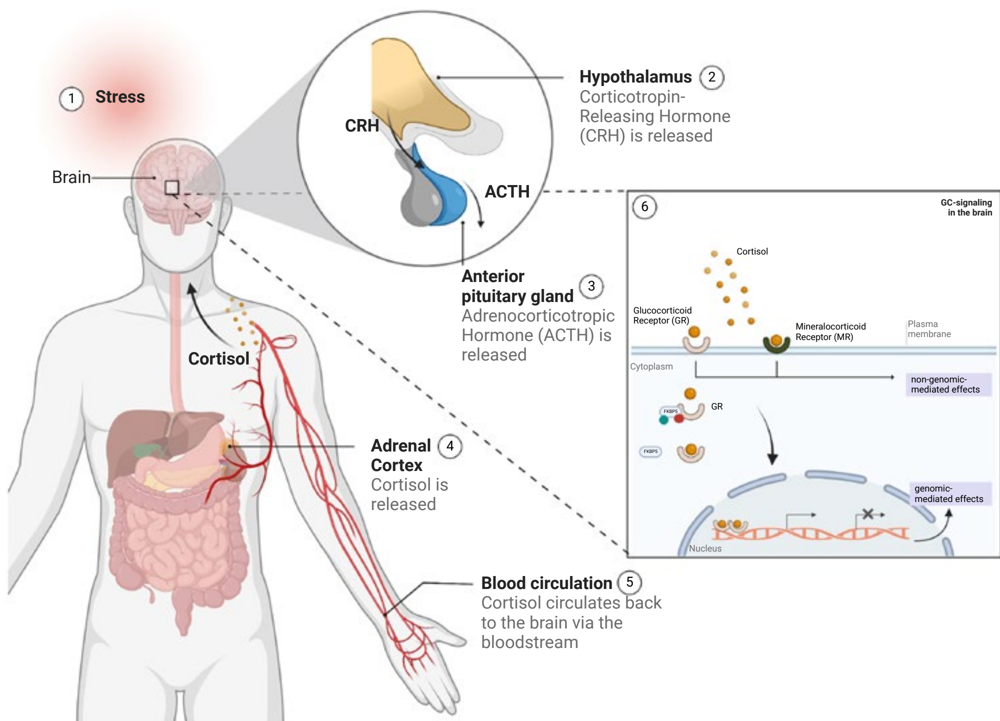


Figure 2. Overview of the stress response in humans following a stress stimulus. (1) Upon the experience of a stress stimulus, (2) the hypothalamus is activated and releases corticotropin releasing hormone (CRH), which leads to the (3) activation of the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH) in the blood stream, followed by (4) the stimulation of the adrenal cortex to release the glucocorticoid cortisol. Cortisol (5) is circulated via the bloodstream to the brain, where (6) it will bind to the glucocorticoid and mineralocorticoid receptors (GR and MR respectively), regulated by gene regulators such as the *FKBP5* and other co-factors, and whose activation will lead to a number of signaling cascades leading to both genomic and non-genomic-mediated effects. (This figure was created with BioRender.)

In vitro options for investigating the neurobiology of GCs

Based on the source of the cells being used, there are a few major groups of *in vitro* techniques that have been used to investigate the neurobiological effects of GCs. In the literature, the majority of *in vitro* studies investigating GC effects are performed on 2D neuronal cultures, which can grossly be categorized in three methodologies. The first and oldest technique is the use of animal primary neural progenitor or neuronal cells harvested from different brain regions[38, 39]. The second corresponds to neuroblastoma cell lines (human or animal immortalized cells that can be differentiated in a neuron-like phenotype) and include cell lines such as the human SH-SY5Y cells[16, 40-42]. The third group entails embryonic stem cell (ESC)- or iPSC-derived heterogeneous neuronal cultures[8, 11, 43, 44]. This category also includes the direct conversion (or trans-differentiation) of adult somatic cells into neuronal cultures[32]. Beyond 2D cultures, the generation of 3D brain models such as cerebral organoids and assembloids has recently gained significant interest[45]. Cerebral organoids can also be generated from ESC or patient-derived iPSCs, are characterized by more relevant heterogeneity of cell types, and capture to some extent the cytoarchitecture of the human brain[46]. Each method carries its own advantages and limitations with some being mentioned in this review[47].

Beyond the challenges

In order to reach the full potential of *in vitro* models in understanding the underlying mechanisms of SRMDs, some important challenges related to investigating the neurobiological effects of GCs *in vitro* must first be overcome. This includes defining *in vitro* stress parameters, identifying and tackling sources of variability in cell models, culture and differentiation protocols and molecular or cellular readouts (Figure 1). Improving *in vitro* GC studies will heavily rely on the development of more standardized protocols and methodologies specific to neuronal cultures and the unique research question, in a way that is not only standardized but also reproducible.

Defining stress parameters *in vitro*

To successfully model aspects of stress mechanisms *in vitro*, an approach that first deals with the semantics of stress and defining certain stress parameters *in vitro*, might be favorable. First it is necessary to define what we mean by *in vitro* models of SRMDs. In the literature, models of stress *in vitro* can refer to metabolic, oxidative, or mechanical stress models[48-50]. However, in the context of SRMDs, an *in vitro* model of stress usually refers to the exposure of a neuronal culture to GCs - e.g., cortisol or synthetic agonists of GC receptors such as dexamethasone, (nor)adrenaline, and/or other mediators of the

in vivo stress response. Just like animal models of stress, *in vitro* exposure to a chemical stressor can be acute or chronic[51]. While acute stress represents exposure to stress for a relatively short amount of time, chronic stress reflects repetitive and/or prolonged stress exposure[51]. Moreover, the effects of (both acute or chronic) stress can be studied shortly after the exposure or after a delayed period of time. In general, there is a lack of consensus as to what defines acute and chronic stress and what defines short-term versus long-term effects. In our opinion, these are important parameters that need to be clearly defined in order to create standardized protocols that can be reproducible and to obtain better *in vitro* model systems to study stress-related mechanisms[52]. Additionally, defining acute and chronic stress might even allow to model and investigate the concepts and molecular mechanisms of allostasis and allostatic load *in vitro*[53], as suggested by McEwen[54] on the use of cultures to examine hormonal interactions, such as mechanisms in allostasis. For instance, acute stress in *in vivo* models is seen as a single exposure to a stimulus that initiates a stress response, and of which the cellular and molecular effects in the brain can be short- or long-lasting[55]. Consequently, an acute *in vitro* GC challenge could be defined as a single exposure to GCs for a short period of time. Defining the latter is difficult as it is unclear how the *in vitro* kinetics and signaling of GCs relate to *in vivo* conditions (a common challenge in cell culture models). In current literature, typical acute exposure times range from hours to 48 hours, which makes it virtually impossible to draw unifying conclusions. In contrast, congruent to *in vivo* conditions, a chronic *in vitro* GC challenge could be understood as a repetitive and prolonged exposure to GCs (e.g., ranging from days to weeks) with a GC-induced phenotype persisting for more than a few days (e.g., more than 72 hours). While it is difficult to setup specific guidelines as to what constitutes an acute and chronic exposures, providing clear descriptions and harmonization of paradigms, will benefit the field to increase the reproducibility of *in vitro* protocols and results.

Sources of variability

Cell models and cell types

A number of different brain cell models have been employed to investigate the effects of GCs on neurons and on different types of glial cells. These included primary cultures, immortalized cell lines, pluripotent stem cell-derived neuronal cultures (2D and 3D), and different types of glial cells, among others. Different cultures introduce a number of variations, first due to the nature of the cell source and, second, related to the different culture mediums used for each culture types, with different supplements including serum. It is important to mention that the presence of GCs in neuronal differentiation media is necessary to drive differentiation *in vitro*[56], which could lead to interferences in assessing the neurobiological effects of GCs added to a culture. Moreover, many of

the differentiation protocols to obtain neuronal cultures are heterogeneous in cell types and many include glial cells[57]. This may in itself influence the response of neurons to GCs knowing that glial cells such as astrocytes have also been shown to respond to GCs *in vitro*[18, 19]. Additionally, a study by Cruceanu et al.[58] demonstrated cell-type specific responses to GCs *in vitro*, with differential-responses between different types of neural progenitors and neurons[59]. Current studies are mainly performed on heterogeneous cultures of neurons, glia, and non-neuronal cells. Future studies should better investigate GC-induced effects in pure neuronal and pure glial cultures (by making use of CPART) such as to facilitate drawing conclusions on the effects of GCs on neurons alone, on neuronal-glial co-cultures, and/or neuronal-glial co-cultures with glial cells pre-treated with GCs. Investigating GC effects in pure cultures alone will allow us to better understand the effects of GCs on distinct cell types that might have a key role in the pathophysiology of certain SRMDs (e.g., serotonergic neurons in MDD). Whereas co-cultures have the advantage of allowing us to investigate the interaction between neurons and glial for example, which more closely resembles *in vivo* processes in normal and pathological conditions.

Important, yet often overlooked parameters, when investigating GC effects *in vitro* are GC receptor expression and GC sensitivity in the examined cells. GC receptor mediated responses are influenced by GC receptor expression on the one hand and sensitivity of the downstream signaling cascades mediated by chaperone and other interacting signaling molecules. Knowing that GR and MR expression differ *in vivo* and *in vitro*, studies should consider expression levels and their ratios of the two receptors in the different cell lines, and results should be interpreted in that context.

For example, Lieberman et al.[31] investigated GC vulnerability in iPSC-derived forebrain neurons from patients carrying an *FKBP5* risk variant and found no effects of dexamethasone, a selective GR agonist, on GR expression in at risk carriers. Their results suggest that low expression of GR in stem cell-derived neurons with a maturation state comparable to fetal neurons[3] might prove challenging to investigate some GC-induced phenotypes. Nevertheless, despite observing no significant changes on neuronal processes such as proliferation and differentiation, GCs may still have an effect on other outcome parameters, and one should be aware of the limitations that the different cell lines carry (i.e., GC receptor levels) which should be considered in the design and setup of their experiments. Advancements in stem cell differentiation protocols and techniques might one day improve the phenotype of the generated neurons and hence improve sensitivity of neuronal cell lines to GCs by expressing GR and MR levels more representative of *in vivo* conditions.

Further, understanding the effects of GC signaling via these receptors separately is essential. GC-induced signaling via GR and MR has differential effects which can be examined using selective agonists and antagonists of each receptor. Understanding these differences may help when comparing cell cultures that differ in GR/MR expression levels. On the other hand, in *in vivo* conditions both receptors work in concert to establish the overall effect of GCs. Studies using endogenous GCs (i.e. cortisol or corticosterone) could be more informative in that respect. Investigators should carefully consider the type of GC to use, and should clearly indicate the rationale in future publications.

Protocols

One source of variability – and a big limitation of *in vitro* studies in general – is experimental variability between different batches of the same cell line[3, 61], or batch-to-batch variability. Another source of variability concerns the face validity of cell lines. For example, neuroblastoma cell lines carry cancerous properties and as such do not reflect the normal growth and differentiation of neurons in culture[62]. Neuroblastoma cells also carry major limitations in their differentiation potential and maturation state. Additionally, rodent primary neuronal cultures can answer a limited number of research questions given their predetermined fate upon harvest.

One way to address these variabilities is to move away from using unreliable cell lines and more towards improved cell models. For instance, patient-derived neuronal cultures have the advantage of investigating genetic-exposure interactions in different possible neuronal identities and in other cell types. Despite several advantages, stem cell technology also suffers from variability in protocols. The use of different protocols to generate (i)PSC-derived neuronal or glial cultures, including the direct and indirect method, also bring about increased variations[3]. For example, Breen et al.[32] observe differential responses to GCs between induced-neurons and iPSC-derived neurons[3]. However, for improved representation of the effects of GCs in humans, one might want to focus on making use of reprogrammed cell lines, and explore the effects of GCs in different neuronal cell types. With the use of reprogrammed cells, individual genomic variation among patients with different genetic background introduces additional variability in the response of neuronal cultures to GC challenges, which should be addressed by using a sufficient number of control- and patient-derived cell lines. Alternatively, the use of isogenic lines could be used to examine the influence of specific genetic variants in relation to GC responses. With batch-to-batch variability being an issue, one need not focus on the use of one cell line only, but instead one could focus the bulk experiments on the most robust cell line, and use other cell lines as validation. Acknowledging the advantages and disadvantages of each model in the initial phases of research design is important in overcoming many of these roadblocks, and in improving standardization of *in vitro* studies.

In addition, a systematic overview of convergent evidence from both animal and *in vitro* models could help identify reliable approaches for investigating GC effects on (non-) neuronal cultures and facilitate a better understanding of different protocols employed, promote exchange of methodologies, and improve standardization.

Assessing GC-induced phenotypes in vitro

While the type of *in vitro* model and the hormone to be investigated are important choices to be made during the design of a study, another challenge is the assessment of the GC-induced phenotype *in vitro* in acute or chronic conditions.

Several readouts have been considered for the detection of a GC-responsive culture such as cytotoxicity and proliferative assays – namely the 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay - but this might not be sufficient. The selection of this readout as an assessment of a GC-induced phenotype is based on *in vivo* studies where increased corticosterone levels lead to cell death and a decrease in proliferation of neuronal cell populations[63]. While these cellular processes explain some of the effects of GCs *in vitro*, they do not assess the full scale of possible GC-induced phenotypes. The colorimetric MTT-assay – most often used as an assay to measure cellular metabolic activity - has also been used in a number of studies as a readout to test different GC concentrations. While a metabolic assay is important in identifying GC effects in cell cultures, it is not reliable as an accurate measurement of cell viability or cytotoxicity and hence has questionable value as a standardized readout for GC effects[64]. It has been reported that the MTT assay suffers from a number of limitations in the interpretation of cell viability and cytotoxicity measures (for an extensive explanation, see[64]), and as such the results of such a colorimetric assay should be followed by complementary assays. While broadly used as a readout to assess effects of different concentrations of a drug and specifically in neuroprotection studies seeking to reverse the negative effects of GCs, its value in assessing neuronal cultures is now questioned, and, instead, flow cytometry assays for cell viability and toxicity are suggested[61]. Moreover, there are doubts whether using MTT assays for assessing the effects of GCs is the best approach in terms of the pathophysiological context of SRMDs, given that cell death is not the major cause of hippocampal atrophy in SRMDs such as MDD and PTSD[65, 66]. For instance, looking into neuronal-specific readouts such as neuronal morphology that relate to e.g., atrophy such as soma size, neurite length, branching and complexity, or even neuronal live-imaging might be preferred means to assess direct GC-effects. That being said, neuronal subtype (i.e., cortical versus hippocampal), research question, and disease etiology or symptomatology, should all be taken into account and used as a justification for performing

MTT assays. For a better assessment of GC-induced phenotypes *in vitro*, some groups have looked instead at the expression levels of a few known glucocorticoid-response element (GRE) containing genes such as *FKBP5*, *TSC22D3*, and *SGK1*[18, 58], which is an improved method in showcasing many of the changes seen following a GC challenge. Moreover, there is an increase in transcriptomic and epigenetic studies (single-cell and bulk) of *in vitro* neuronal cultures following exposure to GCs[32, 67], which may help define hallmarks to assess GC-induced phenotypes in neuronal cultures in the future. It is important to keep in mind, that different *in vitro* models and GC concentrations may bring about different outcomes and will hence make it more challenging to generalize.

Another variation among *in vitro* studies is the wide range of GC concentrations being tested, which hampers drawing solid conclusions from studies presenting contradictory results. This specific concern could be addressed by developing more stringent methodologies for selecting a concentration range that best resembles *in vivo* healthy and non-healthy conditions. In the literature, there has been no attempt to define general criteria for an acceptable concentration range of GCs to be tested, however if we wish to produce standardized and reproducible *in vitro* studies, more research into the influence of different GC concentrations in different cell lines is needed to reduce sources of variability and better model GC effects *in vitro*. It is important to note, that *in vitro* GC concentrations used are relatively much higher than the possible levels in individuals following a stressful experience or in SRMD patients[9]. However, this increased concentration can be justified by the nature of the medium being used, and the presence of certain molecules that breakdown the availability of GCs in culture, hence requiring higher concentrations to reach the required effect[9]. That being said, *in vivo* physiological concentrations might not be a good reference.

Overall, the use of relevant GC-induced phenotypes is important and may depend on the research question at hand. Viability assays, despite being commonly used, are not sufficient to evaluate the effects of GC exposure and should be complemented with expression levels of GC responsive genes and proteins, and/or with measures of neuronal morphology relevant to SRMDs. While it is clear that this is a challenge in and of itself, standardized measurements such as expression of GRE-containing genes, multi-omic data, and using various assessments of cellular morphology to test for concentration ranges of GCs and their effects in central nervous system cells are recommended approaches.

Conclusion

Recent developments instigated progress in modeling stress in-a-dish, although many challenges remain on the road ahead. While many of the challenges may be technical in nature, several equally important ones are more fundamental, especially when it comes to defining stress parameters *in vitro* and selecting the most suited cellular model(s).

It is therefore important to provide sufficient background information and to describe in detail the reasoning behind the selection of a particular cellular model, the type of GC employed, the concentration and exposure time, and the GC-induced phenotype. In addition, authors should be critical of their choices and describe the advantages and limitations of their model, in order for future studies to be improved. Eventually, we foresee that the optimal range of GC concentrations, and criteria for acute and chronic *in vitro* exposures for particular research questions will need to be clearly specified and used across laboratories. Along similar lines, the implementation of robust and more harmonized assessments of GC-induced phenotypes is necessary.

In order to allow for *in vitro* studies to fulfill their full-fledged potential and improve our understanding of stress-related mechanisms in health and disease, it is imperative to tackle these issues. Nevertheless, the invested effort will help in identifying the exact underlying mechanisms contributing to stress susceptibility and resilience, increase our understanding of SRMDs, and may finally lead to new therapeutic strategies.

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Part II

Ethical, Legal, and Societal Implications of Stress Research

Chapter 7

Ethical Implications in Making Use of Human Cerebral Organoids for Investigating Stress-Related Mechanisms and Disorders

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Abstract

The generation of 3D cerebral organoids from human induced pluripotent stem cells, has facilitated the investigation of mechanisms underlying several neuropsychiatric disorders including stress-related disorders, namely major depressive disorder, and post-traumatic stress disorder. Generating human induced pluripotent stem cell-derived neurons, cerebral organoids, and even assembloids (or multi-organoid complexes) can facilitate research into biomarkers for stress susceptibility or resilience and may even bring about advances in personalized medicine and biomarker research for stress-related psychiatric disorders. Nevertheless, cerebral organoid research does not come without its own set of ethical considerations. With increased complexity and resemblance to *in vivo* conditions, discussions of increased moral status for these models are ongoing, including questions about sentience, consciousness, moral status, donor protection, and chimeras. There are, however, unique ethical considerations that arise and are worth looking into in the context of research into stress and stress-related disorders using cerebral organoids. This manuscript provides stress research-specific ethical considerations in the context of cerebral organoid generation and use for research purposes. The use of stress research as a case study here can help inform other practices of *in vitro* studies using brain models with high ethical considerations.

Keywords: stress, chimera, animal research, cerebral organoid, research ethics, informed consent, psychiatric disorders

Introduction

Research into stress and stress-related disorders has long been a focus in ethical discussions, on the one hand due to the harm-prone nature of stress, and on the other hand due to several famous, but today considered unethical, cases of stress research covering for example Philip Zimbardo's Stanford Prison Study and Stanley Milgram's obedience experiments[1]. The ethics of stress research involves several considerations, including the welfare of research participants[2], the use of animals in research, and the potential risks and benefits of the research in question[3]. Inflicting stress in animal and human research, is a well-known problem in research ethics, and is typically considered a harm or burden that needs careful justification and monitoring from a research ethics perspective[4]. One ethical concern in stress research is the welfare of human research participants. Researchers have a responsibility to ensure that participants are informed about the nature of the research and any potential risks, and that they are treated with dignity and respect[5]. This includes obtaining informed consent from participants, ensuring that they are not subjected to any unnecessary harm or discomfort, and protecting their privacy and confidentiality[6]. Another ethical issue, in stress research is the use of animals. Many stress studies involve the use of animal models, such as rodents or nonhuman primates, to study the effects of stress on the brain and behavior[7]. Researchers have a responsibility to ensure that animals used in research are treated humanely and with respect, and to minimize any suffering or harm, hence the existence of ethics committees[8]. This includes providing appropriate housing, food, and care, and using the minimum number of animal necessary to achieve scientific objectives[9], in addition to justifying the worth of the potential scientific goals themselves. Finally, researchers must consider the potential risks and benefits of stress research. While stress research has the potential to lead to new treatments and therapies for stress-related disorders, it is important to carefully weigh the potential risks and benefits of any research study, and to ensure that the research is conducted in an ethical and responsible manner.

Nowadays, investigating aspects of stress and stress mechanisms is possible in human-derived neuronal tissue – without the harm of the donor themselves. Developments in stem cell technology has allowed the differentiation of patient-derived stem cells into both 2-demensional (2D) neuronal cultures and 3D cerebral organoid cultures *in vitro* for the study of underlying mechanisms driving brain development in health and disease[10]. This has the potential to provide an improved understanding of molecular mechanisms involved in neurological and psychiatric disorders[11] such as major depressive disorder (MD)[12] and post-traumatic stress disorder (PTSD)[13-15]. For instance, 3D cerebral organoids, can be used to investigate the respective impact of key stress-related molecules and stress hormones on processes involved in brain development that

are relevant to stress-related disorders, in tissue harvested from different individuals[16]. Thereby, these models, can facilitate research into biomarkers for stress susceptibility or resilience and ideally also for stress-related psychiatric disorders[17] and aim at bringing about advances in personalized medicine, because it makes use of human and ideally patient-specific bodily materials. In addition to this scientific, and potentially clinical advantages, research on stress biomarkers in laboratory stem cell models may prove to be also ethically advantageous and provide more suitable models for human physiological stress-reaction and in some instances serve as replacement for current rodent experiments[18].

Nevertheless, this research into molecular stress-processing does not come without its own set of ethical considerations[19]. With increased complexity and resemblance to *in vivo* conditions, increased moral considerations for these human *in vitro* based brain-models might be warranted. So far, the consensus has been that no specific ethical oversight, by an Institutional Review Board (IRB) for instance, or protection of such models is required. However, this might change in case these models develop even more complexity, the first steps of which can already be seen in 3D cerebral organoids as compared to 2D neuronal cultures. This might raise new research ethical question in studies that if conducted in humans would be considered particularly sensitive, such as studies that intentionally inflict stress and hence harm on participants. Here the question rises on how best to avoid a situation in which the stress-induction will require yet again research ethical attention, because these models are becoming 'too good' and might themselves be harmed in the process of stress research. In addition, new and further questions might arise for tissue donors, increased complexity of and hence a potential need for more vigilance during informed consent procedures, but also issues of reporting research findings back to individual donors might need a place on the ethical agenda of stress-research with cerebral organoids. Finally, as research progresses the creation of chimeric cerebral organoid animals might need special attention if stress-induced research is conducted with them, potentially implying that they might not only have to face the harm of stress-induced research as such, but if somehow 'brain enhanced' they might experience the stress exposure even more seriously than typical experimental animals.

This paper will provide an overview of some of the previously discussed themes, including research ethics, donors and biobanks, and animal chimeras in relation to the ethics of stress research with cerebral organoids. Within each theme, novel ethical considerations that arise in relation to research into stress and stress-related disorders will be identified and discussed. Given the harm-prone nature of inducing stress onto an organism , unique ethical issues, not raised in other organoid research, may arise and hence require special attention. The more complex human cell-derived brain models

such as cerebral organoids become, and the more they start resembling human- or animal-like *in vivo* brains, the more the paradoxical situation might emerge that a new kind of sensitive being is brought into existence, putting us in front of the same challenges that *in vitro* models promised to evade.

Now is a good moment to raise these issues and use the momentum of the ongoing ethical discussions surrounding research ethics of cerebral organoids. Highly complex brain-models have been developed already, hence we are not focusing on a science-fiction field; but to date they have not achieved a level of complexity that could warrant full sensitivity, a kind of consciousness, or any capacities of suffering. Hence, formulating an ethics agenda on the issues to be considered and formulating initial guidance on what it might mean and what is required to proceed ethically in this area of research is a timely endeavor. Unlike currently existing research ethical frameworks for doing science with human and animal participants that have developed only in the aftermath of serious atrocities in medical experimentation[20,21]; proactive thinking about whether, and if so how, we need research ethics for complex brain-models and their biobank infra-structures, might prevent avoidable and unnecessary harm from the outset. The aim of this paper is to sketch the current scene, identify conditions where cerebral organoid research into stress and stress-related disorders does or does not raise specific ethical questions.

Cerebral organoids: Generation and uses

To understand whether a research ethics framework needs to be set for *in vitro* brain-models among which cerebral organoids, and in particular for their research uses into stress mechanisms, we must first understand the nature of what (or whom) we seek to protect. We will describe cerebral organoids, their origin, how they are generated and developed *in vitro*, and the myriad of ways they are currently used and hoped to be used in the future. The current state-of-the-art should help inform us on how best to deal with stress-research with *in vitro* brain models, most notably 2D neuronal stem cells and 3D cerebral organoids.

Cerebral organoids are lab-grown 3D structures that mimic the development of the human brain, with great similarities to the cytoarchitecture, cellular and physiological characteristics of the human brain[22]. Despite their complexity as an *in vitro* model, compared with a real human brain or nervous system, organoids are still rather primitive. However, given their direct linkages to specific individuals, whose somatic cells were used to develop these models, they have increasing research potential with, in the

not-too-distant future, also clinical and personalized applications. Cerebral organoids are defined as "self-organizing 3D tissue" and are generated through a process known as reprogramming, which involves taking cells from a human donor and inducing them to become pluripotent stem cells[23]. Cerebral organoids can originate from a variety of adult somatic cell sources such as connective tissue[24], blood cells[25], or even urine[26] taken from donors. These stem cells are then allowed to differentiate into different types of cells found in the human brain, including neurons and glia. The differentiation of cerebral organoids can either be guided hence leading to specific brain regions (e.g., forebrain, midbrain, or cerebellum), or unguided and hence leading to a self-patterned whole-cerebral organoid with a heterogenous cell population[27]. Guided region-specific cerebral organoids can be further fused to one another, forming assembloids which can further model interaction between different brain regions and to investigate particular research questions looking into communication between different brain regions[28].

Organoid models have been developed to better study developmental processes in various organs and to allow the testing of several drugs and compounds onto human-derived tissues and reduce premature testing in humans. Research conducted with neuronal stem cells and cerebral organoids is not new. By now, they have already been used for different purposes across various research fields and for a variety of medical applications. For instance, in the investigation of several human brain developmental functions[29,30], in health and disease states, to study disease-specific phenotypes of neurodevelopmental disorders[31,32], or to test drugs[33] and different chemical compounds[34]. Cerebral organoids are also gaining increasing attention in the modeling of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases[35]. Moreover, cerebral organoids have already been implanted into rodents in order to investigate their potential in a more complex and vascularized environment, leading to the creation of chimeras[36]. Eventually and in the long term, researchers hope to transplant cerebral organoids into the brain of stroke and epilepsy patients, as a potential treatment strategy to restore brain function[37].

Cerebral organoids, however, have been considered special or significantly different from other organoids since their arrival. The reasons therefore seem mainly to lie in the special status that the brain is given as an organ, as declared by the Nuffield Council[38] and the close association of the brain with who we are as a person, our self and personhood. If similar cognitive functions and capacities could be traced back in the dish, doing research with human derived cerebral organoids might be troublesome and more so than in research with other types of organoids. In so far as this holds for research in general, it is even more applicable in case of potentially harmful research such as research into stress and stress-disorders that makes use of the artificial infliction

of stress and hence harm. In research with human participants this would be bound to clear limits and even then, requires specific justification as to the potential benefit, in terms of knowledge gain, that might result from this research[39]. This raises the question, whether similar questions for stress research should be posed in the context of brain models as well, and if so whether any differences could and should be made between different levels of complexity in these models.

Ethical issues in stress-related research

Diving into the research ethics of making use of cerebral organoids, the following three areas are to be investigated: research ethics frameworks for *in vitro* uses of 3D cerebral organoids, refined and revised biobank research ethics frameworks for the protection of donors, and finally, increased protection for cerebral organoid chimeras.

Research ethics for cerebral organoids

When discussing the ethics of organoids and organoid research, cerebral organoids steal the spotlight. Despite organoid research also carrying more general ethical considerations, there is an inherited belief that cerebral organoids in particular deserve increased moral considerations (that other organoids do not possess) given the nature of the organ (and species) they are modeling: the human brain[40]. That of course is due to the fact that the brain is characterized by unique faculties such as consciousness, sentience, experiencing of suffering and pain, decision-making, and other important higher cognitive functions that contribute to make human beings who they are[41]. Many ethicists believe that if cerebral organoids begin to show increased complexity similar to human brains, then research making use of them must undergo a very similar in-depth ethics review similar to animal or human embryo research ethics reviews in order to ensure that the level of pain or discomfort is minimized, and that methods of experimentation and destruction are refined and appropriate[42,43], especially when paired with other living systems (i.e., chimeras). To date, there are no research ethics guidelines for *in vitro* research models (except guidelines for research into embryo usage), however the generation of cerebral organoids might challenge this situation, calling for a further investigation of the situation and an investigation of whether, why and how, specific ethical considerations and regulations for the use of human-derived cerebral organoids and assembloids for research purposes might be required, especially in cases were harm is exercised, such as for research into stress-related mechanisms and disorders.

There is no doubt that cerebral organoids are characterized by increased complexity when compared to their 2D counterparts (hPSC-derived neuronal cultures). From an

increased heterogenous cell population, to an improved cytoarchitecture, and functional properties, cerebral organoids are to date, an improved model of other *in vitro* brain models out there[44]. Increasing literature on cerebral organoids illustrates their increased ability to respond to different stimuli including the ability to stimulate a skeletal muscle[45], the ability to respond to a stress hormone (e.g., dexamethasone)[46], among others. Additionally, some scientists claim that cerebral organoids exhibit neuronal activity that resembles human fetuses in the first trimester[47]. However, many question these claims due to our little knowledge of brain activity and functioning in human fetuses at this stage of development[48]. One might assume that increased complexity as manifested with an increase in the number of neurons, number of connections, number of cell types, might lead to increased cognitive complexity. However, we know from nature that bigger brains do not necessarily translate to increased intelligence or improved cognitive functions[49]. It is inaccurate to immediately assume that increased complexity in cytoarchitecture will lead to or improve the likelihood of conscious-like signatures in cerebral organoid. There are, and will remain, fundamental differences between human cerebral organoids and human adult brains. These differences constitute fundamental building points for the ability to achieve sentience or consciousness[50]. Moreover, many believe that inducing consciousness requires a highly complex network including a variety of cell types, and sensory inputs that lead to subjective experiences, such as pain and discomfort (which current cerebral organoids do not possess). Importantly, scientists have suggested that the consciousness that contributes to the moral life of human beings can only be manifested with the exposure to social nurturing environments, and the development of language abilities, something that cerebral organoids will never come to develop or even experience[51] (unless depicted in a science-fiction movie). Moreover, cerebral organoids might or might not need be regulated depending on what regions of the brain they are modeling (in some cases of guided differentiation). It could be that certain assembloids might not be morally problematic if the collective assembly of certain organoids (representing certain brain regions) do not lead to the creation of sentience. That being said, should we even be discussing moral justifications for the use of cerebral organoids for research purposes?

Conscious awareness of painful sensations and discomfort is another aspect of conscious experience that is argued when discussing cerebral organoids. To date, that cannot be achieved with cerebral organoids that are developed *in vitro*, and more research is needed to better answer this ethical query[52]. For instance, the brain does not have nociceptors (sensory receptors of painful stimuli) and as such a brain alone will not be able to sense painful stimuli, let alone *in vitro* cerebral organoids. However, some have argued that experiences of stress, sensory deprivation and conscious discomfort might be possible with cerebral organoids[53]. To date, we do not have the technology capable

of assessing these psychological experiences *in vitro*, and we need to be aware that these experiences do not just “emerge” as the organoids grow larger and more complex. Sentience requires a variety of sensory stimuli and the activation of several processes for it to develop, hence being more complex than what is usually portrayed. What we can currently investigate in cerebral organoids are molecular, cellular, and electrophysiological processes underlying particular genetic variants, and/or in response to drugs and hormones for instance. Many philosophers argue that if there is an uncertainty about whether a particular being is sentient, one should not treat it as lacking moral consideration but instead treat them respectfully and as if they have some moral status[54]. However, given the information we currently have on the nature of cerebral organoids, their capabilities and limitations, and in accordance with the consensus, it is safe to say that cerebral organoids are not sentient and hence do not deserve any moral protection.

It is clear what current established research ethics frameworks seek to protect: human research ethics aims to protect humans, and animal research ethics aims to protect animals. The first question that a potential research ethics framework (if required in the future) in the context of *in vitro* brain models will have to answer, however, is what to protect. Should it have to protect: (1) the most complex brain models available, such as current assembloids (consisting of several 3D structures), (2) unguided cerebral organoids with the ability to self-organize with a composition that mostly resembles the developing brain; and/or (3) guided brain organoids that are differentiated into specific regions within the brain. Would oversight and protection be equally needed for different types of cerebral organoid and assembloids? Or would a gradation in level of protection be more adequate, depending on the levels of complexity and maturation level of these 3D models? The term ‘cerebral organoids’ has been used interchangeably in several ethical discussions and analysis; however, a striking difference exists in these different aforementioned 3D models which warrant separate ethical analysis. For instance, an assembloid composed of different brain regions (e.g., hippocampus and hypothalamus) whose combination cannot form sentience, is not deemed ethically problematic, hence would not require any ethical oversight. The same applies for a guided cerebral organoid differentiated into a hippocampal-like structure. Ethical discussions should clearly state the differences between different cerebral organoids[55] and assembloids and point out those that carry ethical implications and those that do not. That would not only improve our understanding of fundamental differences between different cerebral organoids but will also avoid any unnecessary overgeneralization of all research with cerebral organoids. These considerations would also apply for research into stress and stress mechanisms, where making use of less complex 2D or 3D structures might be more justifiable over more complex organoids and assembloids.

Even as we talk about investigating stress mechanisms using cerebral organoids, including mechanisms involved in stress susceptibility, with stress requiring increased consideration in animal and human research ethics frameworks, we do not believe that new research ethical questions arise concerning the need to protect cerebral organoids (or research subjects). *In vitro*, stress itself is not being investigated, however molecular mechanisms involved in stress are. Stress mechanisms have long been investigated *in vitro* using a variety of neuronal cell lines and by exposing the latter to (synthetic) glucocorticoids or other key stress hormones such as noradrenaline. Cerebral organoids have also been used to investigate the effects of a synthetic glucocorticoid called dexamethasone[56]. For example, a better understanding of the mechanisms that drive susceptibility to stress-related disorders could be facilitated through the generation of cerebral organoids from patient biomaterials. In the context of cerebral organoids or *in vitro* research in general, the nature of the stressor being used to induce stress-related response is fundamentally different when compared to *in vivo* research studies on stress where essentially personal experiences of stress and their detrimental effects are playing a role. In vivo, stressors are also of a different type and nature, they are not only neurochemical, but in addition are physical, psychological or social. Current animal stress models make use of these more complex types of stressors. For example, stress in rodent models can be induced by the administration of the stress hormone glucocorticoids (neurochemical), it can be induced using (physical) electric shocks, or even exposed to social stress among other rodents[57]. In the case of cerebral organoids, the stressors in question can only be neurochemical. This situation might change, however, once cerebral organoids were transplanted into rodents, creating chimeras with humanly adapted rodent brains, and specially in cases where the donor is known to be susceptible to stress-related disorders (we will revert to this case later). Given the lack of the capacity to experience stress and be sentient about it in cerebral organoids and given also that stressors *in vitro* are purely neurochemical; *in vitro* stress-related research into cerebral organoids currently, need not be considered as harmful for cerebral organoids. Therefore, it does not require any specific risk-benefit balance, nor does it require a neat justification of any harm or burden inflicted on structures in-the-dish for the sake of research.

Cerebral organoids have reshaped the way we perform neuroscience research, especially when investigating brain development and diseases implicated in brain development. However, these increasingly sophisticated models do not come without their own ethical considerations. Despite no current evidence pushing for an ethical oversight when conducting research with cerebral organoids, we want to reiterate the continuous justification of making use of cerebral organoids, given that they are not here to replace all models (including 2D neuronal cultures or animal models), but are

here as an improved model to answer certain research questions that would otherwise not be easily understood using other less complex models. Reducing the use of cerebral organoids in research in general that does not strictly require their use (but particularly in stress-related research), may avoid getting ever entangled in potential ethical considerations that accompany the use of cerebral organoid in research settings.

Donor-related ethical issues

The current state of cerebral organoid research in fact prioritizes ensuring that adult somatic-cell donors for the generation of cerebral organoids are appropriately protected. For instance, donors might have a legitimate interest in not having their materials used in ways they would potentially dismiss (e.g., dual-use purposes)[58], or they might have a preference to receive knowledge and be informed about any research findings, particularly in case these findings can be linked back to themselves and are potentially meaningful[59]. This raises the questions of whether current legislations on consent for biobanks does still fit with the potential that tissue donated might develop into self-organizing cortical structures[60]. Ethical and responsible practices for the collection of patient or human biomaterial (including somatic cells) include transparent disclosure of the benefits versus risk of participation in the research study, in addition to short- or long-term goals of the study in question, as advised by the International Society for Stem Cell Research (ISSCR)[61]. In the case of cerebral organoids, this might include actively engaging the potential participants in the informed consent process (and participant-appropriate alternatives in case of vulnerable groups including children or individuals with cognitive disabilities), clearly informing potential donors that genetically-matched cerebral organoids will be generated possibly unraveling peculiar medical-related information about the donor in question, and finally, ensuring that no false hope is transmitted to the donor about directly benefiting from donating their biomaterials for research[62]. Additionally, many limitations remain in that first, these guidelines are not law-abiding and as such do not strictly prevent malpractices; second, the generation of cerebral organoids (or other iPS-derived cells and organoids) is rather challenging when it concerns the use of samples from biobanks and whether tissue bank donors are aware, comfortable with participating, or whether they may even opt out from (future) cerebral organoid-related research[63].

We do see a peculiar ethical challenge here, in the sense that mainstream media might also influence the informed consent procedure, especially in situations where the research in question relates to investigating stress mechanisms *in vitro* which involves exposing cerebral organoids to a chemical stressor[64]. With the COVID-19 pandemic, it became clear how fast medical misinformation can spread, and how severe the consequences can be[65]. The overturning of Roe v Wade has also challenged the stance of the

scientific community in informing policy and law[66]. Stress research, and ethics of stress research, has always been a sensitive topic, particularly in the context of both human and animal research as we will discuss in the coming section. With a growing number of non-scientific publications reporting research into cerebral organoids[67], we believe caution should be exercised in the framing and communication of research into cerebral organoids to the public and potential donor participants. The public communication should actively countervail the impression that generating and making use of cerebral organoids to investigate stress-related mechanisms would translate into somehow stressing and hence harming cerebral organoids that are exhibiting signs of sentience. That of course raises considerations as to how research into cerebral organoid must be communicated, including questions on which information should be provided on currently intended research, on research not drafted yet but potentially planned and conducted in the future, but also how detailed should the information about potential sentience or other ethical considerations raised by cerebral organoid generation be. How should and could these issues be discussed with potential donors, in a way that provides them with relevant information to make up their minds on donation in a reasonable way but does not encourage or discourage them to donate their bodily materials for the wrong reasons.

Additionally, in the case of stress-related research, researchers could identify an increased vulnerability to stress-related disorders through screening the cerebral organoids for (epi)genetic variances that have been shown to be associated with increased susceptibility (or even resilience) to stress-related disorders. Identifying susceptibility or resilience to stress-related disorders can also be accompanied with its own set of ethical implications, as it has been previously described[68,69]. Therefore, reporting back to donors about predicted susceptibility or resilience and communicating with them the meaning of such findings is questionable and requires careful thought.

This in turn, invites us to draft guidelines for the ethical communication of cerebral organoid findings for scientists, journalists, science communicators, and other professionals that are involved in the dissemination of findings related to cerebral organoid research in general. This could improve the public understanding of cerebral organoid-related findings without communicating false hope or hype to the general public.

Beyond organoids in-a-dish

While cerebral organoids in the dish have raised many ethical questions, further and potentially new and more serious questions might arise if these organoids were no longer kept in an artificial environment but transplanted into a more natural

environment. Cerebral organoids are cultured in a dish where they essentially form an island detached rather than connected to a body, but they can also be transplanted into actual living beings. Currently, this has been performed by researchers who have transferred human cerebral organoids into the brain of rodents and thereby have created humanly adapted chimeras[70]. Performing research - and particularly in the context of stress-related research with such chimera's, raises further ethical questions that did not arise in the same way when cerebral organoids were developed and cultured in the dish. For example, the mere transplantation of human cerebral organoids into rodent brains is an invasive procedure and often leads to the rejection of the transplanted organoid, the formation of tumors, faulty integration into the host, and other possible complications that directly harm the receiving animal. While this concern holds in general for the creation of all kinds of chimeric animals, in the current context the additional question arises on how research into stress and stress-related disorders using cerebral organoid chimeras would impact the welfare of hosting animals as compared to non-chimeric animals? While research on cerebral organoid chimeras raises several ethical questions as such, we believe that stress research with cerebral organoid chimeras raise additional and more specific ethical questions.

In order to tackle these questions and in particular questions on the justifiability of stress-research in chimeric animals, we should revert to debates in research animal ethics. Since the beginning of the 1980s, the use of animals for research purposes saw a decline with increased public advocacy, awareness among scientists and the introduction of regulations on animal use. This was accompanied by the implementation of animal ethics committees and a relative improvement in the quality and use of research animals[71,72]. In Europe for instance, according to the directive 2010/63/EU[73], the performance of animal research must be preceded by an ethical approval by a competent authority. The movement towards the ethical use of animals in research, was also inspired by William Russell and Rex Butch and their 3R framework – replace, reduce, and refine. This framework urged scientists to replace animals with alternative models or at least with "lower" species; reduce the sample size of animals by including not more than the minimum number needed for statistical significance; and finally, refine the experimental conditions by minimizing experiences of pain and suffering, in addition to improving quality of care such as housing facilities, and welfare[74]. These developments have improved the use of animals in research in a way that ethical justifications of the use of animals for research purposes became a requirement[75]. However, with issues concerning reproducibility and their translational ability to the bedside, the validity of animal models for answering some research questions into human health and disease is becoming increasingly questionable[76], particularly in the context of psychiatric or mental health issues that relate to human behavior and experiences. Ethical doubts

about the justifiability of animal research have further intensified since the advent of organoid research and its great promises in several avenues including personalized medicine, toxicology, drug testing, and improved modeling of human disorders, making them particularly attractive as a suitable alternative to animal research[77].

Research animals have long been used for the investigation of a number of disorders, even specific disease models have been developed to better represent the underlying pathology. When it comes to stress disorders, by today a number of different stress animal models for major depression, anxiety and PTSD have been developed. These models cover neurochemical models that induce stress by corticosterone treatment or neuroinflammation. Given the fact that animals unlike organoids are also social beings, stress-models in animals do also cover social forms of stress such as early maternal separation, social defeat, social isolation, chronic unpredictable stress, or forms of learned helplessness[78]. The nature of the stressor is also of interest here, while *in vitro* stressors relevant to stress-related disorders are mainly of a chemical nature, in chimeric animals of cerebral organoids, this limit is no longer needed and stressors theoretically could include chemical, physical, and psychosocial features. The use of animals for stress experiments is itself an ethical concern due to the harm and discomfort to which these animals are subjected. To put it more clearly, the conscious experience and conscious suffering caused by the stressor is of particular interest to researchers because that ensures the validity of their model. And without this conscious suffering, animal stress models are of no relevance for researchers investigating stress in the lab. This raises questions on the harm-benefit balance and the requirements one may put on the relevance of the knowledge gain that might be achieved with such experiments such that it can be proportionate with the harm inflicted. This conscious suffering is also measurable in the form of behavioral tests (including the sucrose preference test, the open-field test, among others). For example, an animal that shows stress symptoms would score low on the sucrose preference test, as compared to a non-stressed animal. Scientists measure stress effects on animals also by using behavioral output measures such as anhedonia, assuming that animals that exhibit more anhedonia are more stressed than those that show relatively less signs of anhedonia. The use of cerebral organoid chimeras may raise ethical questions as to the meaning and hence justifiability of the suffering inflicted by stress-models in both the animals in question, and the implanted cerebral organoids.

Today, it has been shown that implanted cerebral organoids integrate with the vascular and nervous system of the host animal and thereby, increases complexity of the organoid in several ways[79]. This raises question of whether and how cerebral organoids transplanted into an animal initiate a humanization of the animal, making it more human and

therewith potentially also more protection worthy compared to non-chimeric animals. Related to this, there are growing concerns that introducing human neuronal tissue into animal brains might lead to the development of human-like characteristics, such as self-consciousness, and improved cognitive abilities[80,81]. In so far as these concerns prove reasonable, they would imply that stress research with such chimeric animals would be even harder, or maybe impossible, to justify than similar research with 'typical' experimental animals. However, the concept of humanization of cerebral organoid chimeras has been argued against[82,83], even considered less constructive, first because this has not been demonstrated through behavioral tests in chimera, and second due to other more eminent problems being put forth as more urgent in relation to cerebral organoid research and transplantation, including the welfare of chimeric animals[84].

Nevertheless, instead of claiming that cerebral organoid chimeras are becoming more human-like in general, another perspective has been put forth concerning chimeric animals transplanted with cerebral organoids, and that is brain enhancement of cerebral organoid chimeras[85]. Brain enhancement in this context ranges for example, from chimeric cerebral organoid animals exhibiting increased reaction times, improved visual functions, ameliorated learning and memory functions, to self-awareness and meta-cognition capabilities.

With the possibility of brain enhancement, a decrease in the welfare of the animals might be at stake beyond the negative effects currently being reported of the transplantation itself. While in certain contexts, cerebral organoid chimeras may lead to brain enhancement, in other cases, such as stress-related research, cerebral organoid chimeras may lead to brain-induced vulnerabilities and susceptibilities to stress-related pathologies, as suggested by H Isaac Chen et al. (2019)[86]. For example, humans and nonhuman primates, are known to be increasingly susceptible to the negative effects of stress and exposure to stress stimuli, when compared to rodents and other vertebrates[87]. Therefore, stress-research with brain organoid chimeric rodents can be considered a double-edged sword. On the one hand, a model that more likely resembles vulnerabilities seen in humans might increase the validity of the animal model and overcome, or at least reduce, current criticisms that argue that animal models for investigating stress-related disorders lack validity and have only poor, if any, reproducibility in humans. On the other hand, however, this very potential advantage also carries the chance of increased negative consequences for the welfare of the cerebral organoid chimeric rodent and lead to increased depressive-like symptoms that they otherwise are unable to experience. This raises the question on whether new research ethical guidelines on cerebral organoid chimera should be developed and whether these should pay particular attention to, or maybe even ban certain kinds of, stress research that might inflict particular suffering on

chimeric animals, even though it remains unclear which precise knowledge gain might result from this intensified suffering. However, before such guidelines could ever be developed, first research is needed that identifies what could count as ethical handling of cerebral organoid chimeras and what it would require. If an improved understanding of the identity and status of such chimeras was available, including ways to attain to their welfare and the limits to their handling should be respected, then guidelines might be developed on how to realize the requested level of protection potentially considering the kind of host animal and the kind of stressor (neurochemical, physical, psychological or social) intended to be used in any study.

Conclusion

Undeniably, cerebral organoids offer novel and exciting opportunities for the understanding of brain development in healthy state and in the context of neurological and psychiatric disorders. However, the capacities and promises of cerebral organoids are not limitless (both in case they exhibit or do not exhibit sentient-like features). And it is clear that the use of cerebral organoids does not come without moral considerations, in fact they raise several unique ethical questions, especially in the context of stress-related disorders. In their current form today, the use of cerebral organoids for research purposes is not ethically problematic from a research ethics perspective, meaning that the potential benefits and knowledge gain resulting outweigh the risks or harm for the organoid itself. Donor-related ethical issues including communication of the promises, limitations, questions on reporting back research findings on stress susceptibility and resilience, next to issues about the proper protection and information of donors, are issues that require increased attention and consideration. Ethical debates on stress-research in brain models should also pay particular attention to the welfare of chimeric animals with cerebral organoids, because they might experience increased harm and suffering. These ethical considerations of cerebral organoids are more pressing on the ethics agenda than the potential protection worthiness of cerebral organoids.

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7

Chapter 8

Biomarkers for PTSD Susceptibility and Resilience, Ethical Issues

Based on publication:

Bassil, K., Rutten, B.P.F., & Horstkötter, D.(2019). Biomarkers for PTSD susceptibility and resilience, ethical issues. AJOB Neuroscience 10, no.3 (2019): 122-124.

Expanding on the International Neuroethics Society Emerging Issues Task Force comments on military neurotechnologies (Kellmeyer et al. for the Emerging Issues Task Force, International Neuroethics Society 2019), we elaborate on the ethical questions that arise when applying neuroscience findings of biomarkers to the prevention of posttraumatic stress disorder (PTSD) among members of law enforcement agencies.

PTSD is a highly debilitating mental disorder that impacts not only the health, social life, and economic situation of those affected, but also the well-being of their families. Occasionally, PTSD may also have an influence on the safety of the wider communities, particularly in cases where PTSD patients display aggression and/or violent behaviors toward others.

Resilience and susceptibility in PTSD

Being a stressor-related disorder, PTSD is closely linked to the exposure to shocking and/or life-threatening events. However, after being exposed to such an event, a substantial number of individuals do not develop PTSD or other mental disorders. Thus, substantial interindividual differences exist in the response to a traumatic exposure. Such differences are commonly differentiated in a dichotomized way between individuals who do not develop mental disorders and are considered resilient, and those who do develop a mental disorder and are considered susceptible (Yehuda 2004). Members of law enforcement agencies, like the police or the military, are particularly at risk to develop PTSD, because of the nature of their profession, which is characterized by dealing with serious incidents such as traffic accidents, (mass) shootings, armed threats, or war combat. Incidence rates of PTSD in populations of military personnel and police officers are substantially higher (Weichselbaum et al. 2017) than in the general population.

Today, the underlying pathophysiology and etiology of PTSD are not yet completely understood, although it has become clear that PTSD is associated with alterations in multiple biological systems working in concert and impacting a range of brain and physiological functions (Daskalakis et al. 2018). A series of studies has explored the benefits of making use of potential biomarkers as identified by brain imaging, behavioral and cognitive measures, and measurements of molecules bathing in peripheral biofluids including blood, urine, and saliva, to better understand the occurrence of PTSD (Schmidt et al. 2013). Also, our research group has identified candidate biomarkers in a military cohort, including differentially methylated genes. We have also obtained evidence showing that changes in DNA methylation in certain genes may be linked with changes in clinical PTSD symptomatology, thus suggesting that distinct epigenetic marks may differentiate susceptible versus resilient individuals (Rutten et al. 2018).

While two of us (KB and BR) are engaging in the basic and translational neuroscience of PTSD, we all believe that this upcoming possibility to predict resilient and susceptible individuals even before the exposure to traumatic events can trigger a series of ethical questions. These should be addressed proactively, that is, before the very occurrence of actual applications, in order to guide responsible decision making and to raise awareness about salient ethical issues in biomedical PTSD treatment and prevention.

Today, ethical studies and discussions on biomarker research in trauma-related mental disorders including PTSD are rather sparse. Where they do exist, the scientific reports seem to mostly focus on research-related ethical questions (Jain et al. 2011), on questions that arise when treating PTSD patients (Yang et al. 2017), and on ethical issues relevant for the criminal justice system (Soltis et al. 2014). However, biomedical research on PTSD susceptibility and resilience furthermore raises ethical questions in the context of prevention that have been largely underrepresented in the scientific literature. Also, the INS Task Force focuses on different issues such as neuroweapons when considering neuroethics in the context of the military and law enforcement agencies (Kellmeyer et al. 2019).

Prior to setting the agenda, we acknowledge that despite the use of increasingly sophisticated techniques to differentiate between susceptible and resilient individuals, this is not a black-and-white issue. Absolute resilient or susceptible individuals are likely the exception rather than the rule, and most people will end up somewhere along this spectrum. Furthermore, the phenotype of resilience is dynamic and may change during life. Still, for the time being, we prefer to structure the ethical debate around the two dichotomized ideal types, because they allow us to structure a complex future reality and to facilitate clear ethical thinking.

PTSD susceptible individuals: ethical issues

Moral failure

The prevention of PTSD is particularly important, and a failure to apply and translate upcoming insights can be considered a moral failure, because we will be allowing the manifestation of avoidable harm and suffering. That is, as soon as biomarkers, with some accuracy, allow the identification of susceptible individuals even before exposure to any traumatic experience, we should reconsider how responsible and justifiable it would be to let these individuals run enlarged risks of experiencing traumatic events. While it might not be possible to avoid such experiences over a person's life span, it seems very possible to avoid enlarged risks to exposure, as in police or military contexts. Having such findings and not using them for risk prevention purposes could be considered an unethical practice, because it generates avoidable cases of PTSD.

Susceptibility-informed policies

In this sense, it should be considered whether and how these biomarker-based findings should influence legislation and policymaking. For example, should screenings for PTSD susceptibility precede military and police recruitment and deployment to war combat and crime scenes, respectively? Such strategies might prevent susceptible individuals from being presented with traumatic experiences. As such, biomedically informed recruitments could avoid, or at least reduce, the very occurrence of PTSD among members of law enforcement agencies. However, is this a desirable situation? Early identification of susceptible individuals—that is, before the very occurrence of situations that trigger the onset of the disorder—does also lead to some critical considerations. Would such screenings be obligatory during recruitment? How will susceptible individuals be governed? Will they also face stigmatization and social or professional exclusion or discrimination? Will they be denied job opportunities? But also, fundamentally, are PTSD susceptible individuals, purely by underlying biological sensitivities, different from those with a different genetic and/or neurophysiological makeup? Will they come to perceive themselves differently? How will they be seen by their peers? To date, these are open yet fundamental questions, which we should ask and find answers to in order to proceed in ethically and socially responsible ways in our search for PTSD biomarkers and for our ultimate aim to render these clinically relevant.

PTSD resilient individuals: more ethical issues

Dual-use

At this point, the potential application of biomarkerbased PTSD research also gives rise to specific concerns about dual use. The dual-use aspect of military neurotechnology holds true not only for neurotechnological arms race and what is termed "neuroweapons" as identified by the INS Task Force. Identifying PTSD-susceptible individuals necessarily goes together with detecting those who are resilient. Today, it is unclear what this knowledge might imply for those concerned, as well as for their current or future employers. What does it mean to know that you are resilient to PTSD, when being on a military mission or when visiting a highly-troublesome crime scene? What does it mean that your employee knows this about you? Will those who know that they are more resilient, be desensitized to real-life violence and the suffering of others? Will they be more willing to participate in more violent and de-humanizing behaviors? On the other hand, the situation might be that those considered PTSD resilient will more easily than others be sent to particularly dangerous situations.

The end of guilt?

A final point relates to the feelings of guilt and shame, frequently reported by those who do suffer from PTSD, partly due to behavior committed by themselves, but later regretted. For example, Yang et al. (2017) report about an army reservist who later learned he had killed a young child among adult combatants. This soldier suffered from PTSD and had clear feelings of "guilt, shame, anger, irritability, intrusive thoughts and nightmares" (435). What does it mean to feel guilty about one's own behavior, or about unintended bad consequences of one's own actions? In a certain way, feeling guilty of some wrongdoing—in this case, killing a child during combat—is of significant ethical worth. At least, doing wrong and not feeling guilty can be considered to constitute a moral failure. But how will feelings of guilt change after identifying individuals as being PTSD resilient? Will they lose, or fail to have, the capacity to feel guilty or shameful for any wrongful behavior? This certainly requires further thought, particularly on the relationship of such moral emotions with psychiatric disorders (Fontenelle, de Oliveira-Souza, and Moll 2015).

Conclusion

The questions presented here are open and unexplored to date. However, as we go along, developing neurotechnologies that will enable us to differentiate between PTSD susceptible and resilient individuals on the basis of any (including genetic, neurophysiological, and/or clinical) markers, it is of great ethical value to think these issues through before the technologies become available.

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8

Chapter 9

General Discussion

This thesis provides a deeper understanding into potential mechanisms involved in stress-related disorders (SRDs) by using a human *in vitro* model and introduces novel ways to model stress mechanisms for investigating biomarkers of SRDs, including post-traumatic stress disorder (PTSD). In this chapter, the first four research questions on modeling stress mechanisms *in vitro* will be discussed, followed by the final research question on the ethical implications of this line of research. Finally, an overview of the main limitations of the different chapters will be provided, ending with future perspectives for the field.

Research question 1: What are the neurobiological mechanisms following glucocorticoid exposure that are investigated *in vitro*?

As discussed in **Chapter 2**, *in vitro* models of stress allow the investigation of the effects of key stress hormones, independently or in combination, on underlying mechanisms and pathways implicated in SRDs. *In vitro* stress research aims to answer the question of how cellular, molecular, and (electro)physiological processes are altered following exposure to stress hormones, as opposed to what behavioral outcomes are altered following stress events, which is a main objective in *in vivo* models. In doing so, we move away from examining the overall impact of stress and instead, we can perform experimental studies that involve targeted manipulations on specific cells, at specific developmental stages, and within defined contexts. Although this approach has both advantages and drawbacks, it plays a critical role in generating and validating novel research findings that contribute to our improved understanding of the molecular and cellular mechanisms underlying the response to stress.

The response to stress is (at least in part) mediated by glucocorticoids (GCs). GCs are steroid hormones that are known to be involved in several physiological processes including development, inflammation, and cognition by binding and activating GC receptors. In the brain, GCs induce both genomic and non-genomic mediated changes that influence mechanisms impacted in SRDs and are responsible for the negative feedback regulation of their own synthesis and release. Given the adverse effects of GCs on neuronal development and functioning investigating GC effects *in vitro* is considered essential to better understand the etiology of SRDs[1].

To date, *in vitro* studies are performed on a number of different cell lines including (i) primary neuronal cultures, (ii) *ex-vivo* brain slices, (iii) animal or human neuroblastoma cell lines, and more recently (iv) 2D and (v) 3D pluripotent stem cell (PSC)-derived

neuronal models (including embryonic and induced PSC (iPSC) lines). *In vitro* studies looking into the neurobiological effects of GCs focused on (i) genetic and epigenetic variations, (ii) molecular mechanisms, (iii) cellular processes, (iv) glial cells, and (v) neuro-transmitter systems implicated in SRDs, such as the serotonergic system[2, 3].

The current literature on *in vitro* GC effects points to a lack of standardization in GC exposure paradigms, which hampers proper scientific study of the neurobiology of GCs. While many studies aim to investigate the underlying mechanisms of GCs, others make use of *in vitro* studies as a validation for *in vivo* findings on the effects of stress, and a smaller number of studies aim to test the neuroprotective effects of drugs or nutritional supplements on GC-induced cytotoxicity. Dysregulation of neural progenitor cell (NPC) proliferation, survival, and neuronal differentiation is most often reported in several *in vitro* studies[4]. However, as the literature demonstrates a great variation in experimental conditions, it is not surprising that results remain conflicting.

It is clear that GCs negatively impact different aspects of neuronal development and functioning, including neural differentiation. However, the reported findings highlight a few open unanswered questions that need to be addressed by researchers in the field of GC and SRD research. For instance, what is the role of GCs as mediators of the effects caused by different types of stress? For example, it is well established at this point that acute stress brings about different outcomes than chronic stress or traumatic stress. It is also clear that the effects of acute versus chronic GC exposure leads to a differential response in central nervous system cells, with chronic stress causing increased negative alterations in important processes. Nevertheless, the importance of GCs as mediators of the effects of acute and chronic stress on the mechanisms underlying risk to SRDs remains elusive, and further mechanistic studies are required to address this issue.

It is clear that GCs negatively impact a number of central nervous system cell types including different types of neurons and glia. The impact of excessive and chronic GC concentrations seems to impact different types of neurons at different stages of neuronal development, as much as glial cells, which is well characterized both in studies using *in vivo* and *in vitro* stress models. More extensive research is required, including research on complex brain models and co-cultures, to better understand whether different cell (sub) types have differential vulnerability for lasting effects of GC exposure.

The underlying GC-mediated neurobiological mechanisms of stress susceptibility and resilience are not well understood. Several biomarkers have been identified categorizing individuals as susceptible or resilient to SRDs, including PTSD. To better capture these underlying differences, studies making use of human iPSC models from SRD patients and controls or for example in cohorts of PTSD resilient and PTSD susceptible individuals, can

provide important new insights on the molecular and cellular underpinnings of differential susceptibility to the effects of stress.

A major criticism related to the use of *in vitro* models to examine the effects of stress, revolves around the reductionist approach, e.g., by investigating solely, the neurobiological effects of GCs. The stress response involves an interplay between different physiological systems including the hypothalamic-pituitary-adrenal axis and the autonomic nervous system. This entails additional hormones, in particular noradrenaline (NA), which has also been implicated in SRDs, such as PTSD, anxiety, and major depression disorder (MDD). A better understanding of the interaction of GCs with other stress hormones is crucial for improving the validity of *in vitro* models on the one hand, and for a better understanding of stress mechanisms implicated in SRDs on the other.

That being said, *in vitro* paradigms – employing GC exposure alone or in combination with other stress hormones – could be used to shed light on the distinct neurobiological mechanisms of different types of SRDs. Various *in vitro* models investigating GC effects, have been employed to model aspects of SRDs, without necessarily differentiating between specific types of SRDs, e.g., PTSD and MDD. For example, HPA axis dysregulation in PTSD and MDD is different, with PTSD being characterized by a hyper-suppression whereas MDD being characterized by a hypo-suppression. It remains to be determined what are the optimal *in vitro* conditions for accurately modeling different SRDs.

Research question 2: What are the neurobiological effects of chronic cortisol in human cortical neurons throughout neuronal development?

Our *in vitro* model shows that CORT exposure has a negative impact on different stages of neuronal development, which may translate to increased vulnerability to SRDs in later life. In this respect, I have identified specific molecular players that are sensitive to CORT, which form significant focus points for future research, including *in vitro* or other approaches. Moreover, the transcriptomic trajectory profile may be informative for future *in vitro* paradigms of neurodevelopment-related disorders.

In **Chapter 3**, I examined the effects of chronic cortisol (CORT) at different stage of neuronal development in human embryonic stem cell (ESC)-derived cortical neurons. Using this paradigm, I observed a decrease in proliferation and survival of NPCs. There was no change in overall differentiation, but a decrease in *Pax6* expression was noted in young immature differentiating neurons, possibly indicating that CORT attenuates

molecular processes of differentiation. Moreover, I observed an increase in expression of the post-synaptic marker PSD-95 and a decrease in expression of the astroglia marker *GFAP*, along with a decrease in neuronal activity in differentiated maturing neurons. To assess whether chronic CORT exposure led to unique GC responsive genes in each stage of neurodevelopment, I analyzed differentially expressed genes (DEGs) from a whole transcriptome assessment. We identified 519 unique DEGs in NPCs, 217 in young immature differentiating neurons, and 285 in maturing neurons. Many of the top DEGs have been previously reported to be either responsive to GCs or implicated in stress and SRDs. For instance, *SERPINE1*, which is involved in immune-related functions, was significantly upregulated in NPCs following chronic CORT, and has been previously shown to be upregulated in human iPSC-derived astrocytes of MDD patients following chronic CORT exposure[5]. In young immature differentiating neurons, upregulation of *KLF9*, a GC-responsive gene was reported. And finally, in maturing neurons, an upregulation of *CCN2*, a gene involved in stress susceptibility was observed.

Of the stage-specific unique DEGs, I performed meaningful pathway and GO enrichment analyses for each stage. This revealed stage-specific pathways involved in synaptic signaling in NPCs, extracellular matrix organization in differentiating neurons, and transmembrane receptor protein serine/threonine kinase signaling pathway in maturing neurons. Some of these implicated pathways have also been reported in SRDs including MDD and PTSD, which partially explain the associated cognitive disturbances in these mental disorders. Together, the findings presented in this chapter provide preliminary evidence for the unique stage-specific chronic effects of CORT on mechanisms previously implicated in SRDs, in human cortical neurons.

Chapter 4 presents one of the first trajectory analysis of the transcriptomic signature of chronic GC effects in human cortical neurons. We opted for identifying gene patterns that drive differentiation of cortical neurons through trajectory analysis, and then sought to investigate CORT-induced effects on these genes by looking at changes either during early or later stages of differentiation. We identified 8 distinct patterns driving neuronal differentiation in control samples. Of these genes, 34 were affected by chronic CORT in early stages of differentiation, and 47 in late stages of differentiation. The top significant genes included *LRRTM2* and *TSPAN5* in early stages, and *KCND3*, *KCNIP4*, and *GRIA3* in late stages. Interestingly, these CORT-sensitive genes are known to be involved in neuronal differentiation and synaptic plasticity related pathways, highlighting the relevance of CORT-induced effects on neuronal differentiation and maturation known to be involved in SRDs. For a better understanding of the relationship between different CORT-sensitive genes in early and late stages of differentiation, I performed gene-gene network analysis. Interestingly, the top significant genes in early stages of differentiation

were connected to one another through genes which have been demonstrated to play a role in neuronal processes including differentiation and synaptic plasticity, in addition to cellular stress-associated mechanisms. Whereas late differentiation genes were interconnected through genes involved in stress-related behavioral changes and psychiatric disorders.

Since common polygenic risk is linked to psychiatric disorders, including MDD, PTSD, and schizophrenia, I assessed whether SNPs implicated in these disorders are significantly represented in our identified CORT-sensitive genes. Interestingly, three of these genes showed robust co-localization with genes which have previously been linked to schizophrenia, a neurodevelopmental disorder. Moreover, *PCDHA12* was recently identified as a variable methylated probe, in a twin study investigating DNA methylation changes in early-onset MDD[6]. These findings point to the involvement of neurodevelopmental genes in stress-related mechanisms and SRDs and confirm the implication of previously identified extracellular matrix and cell-cell adhesion genes.

Together, these preliminary findings indicate that specific genes and pathways involved in neuronal differentiation, maturation, and synaptic plasticity may be implicated in GC-related mechanisms known to be dysregulated in SRDs. They also highlight stage-specific effects, suggesting that some stages, such as for example the NPC stage, may exhibit increased sensitivity to CORT effects. For instance, it has been demonstrated that cerebral organoids, characterized by a heterogenous cell population, lead to cell-type-specific responses to GCs.

Furthermore, while I observe stage-specific responses to chronic CORT in **Chapter 3**, the findings of the trajectory analysis in **Chapter 4**, highlight that CORT-responsive genes, such as *LRRTM2*, in early stages of differentiation may also be implicated in later stages of differentiation, suggesting possible carryover effects, with certain genes exhibiting CORT sensitivity throughout different stages of differentiation. Two distinct methodologies are provided to model different aspects of GC effects, with **Chapter 3** highlighting cell-type-specific responses, and **Chapter 4** focusing on modeling the continuous trajectory of neuronal differentiation. Together, these results allow for future functional studies to gain a better understanding of the role and function of these mechanisms and pathways in stress susceptibility and associated disorders.

Our findings raise important questions about for instance, the validity of our model and the chronic CORT paradigm. The negative alterations caused by chronic CORT at the different stages of neuronal development is supported by both *in vitro* and *in vivo* studies investigating effects of GC in neurons, and their association with underlying mechanisms associated with SRDs.

Moreover, our findings from **Chapter 3** and **Chapter 4** prompt the question of whether this model, which can capture certain aspects of neurodevelopment, is better suited for modeling neurodevelopmental disorders, such as schizophrenia, which are associated with exposure to stress and particularly excessive GCs, rather than SRDs that manifest in adulthood, such as MDD and PTSD. Although we recognize that early life stress (ELS) is a risk factor for SRDs and other neurodevelopmental disorders like schizophrenia, our model may more accurately reflect different aspects of neurodevelopmental disorders.

Research question 3: Could an *in vitro* model of ELS be used for investigating candidate genes involved in stress susceptibility?

To understand the effects of stress exposure on candidate genes that have previously been associated with PTSD susceptibility in a military cohort, I utilized a chronic CORT exposure paradigm in human ESC-derived cortical maturing neurons, as described in **Chapter 3**. We examined the effects of CORT on DUSP22 and ZFP57, which are known to be involved in PTSD susceptibility, as detailed in **Chapter 5**. Our exploratory study revealed that these candidate genes were responsive to chronic CORT exposure *in vitro*, resulting in changes in DNA methylation (*DUSP22*) and expression (*ZFP57*).

For a molecular signature or gene to be a robust biomarker candidate, there are a number of criteria required, including a high specificity and sensitivity, among others[7]. To date, there is not enough evidence in the involvement of these genes in SRDs and underlying mechanisms, and more fundamental research is required to label these genes as diagnostic biomarkers for PTSD susceptibility. Here, I take the first attempt to investigate the effects of chronic CORT exposure on the expression of these genes and their targets, and demonstrate CORT responsiveness of these candidate genes, which validates to some extent the previous findings of their involvement in PTSD susceptibility[8]. In addition, this is a first proof validating our *in vitro* CORT model and showcasing that it can be utilized to verify findings from large scale human omics studies and to explore the role of candidate genes in SRDs, specifically highlighting their involvement in stress susceptibility mechanisms rather than trauma-induced effects. While this research only scratches the surface, it provides an avenue to further explore the role and function of these genes (and other candidate genes) in relation to neuronal processes first, and then in response to GC treatment.

Research question 4: What are the challenges and future considerations for improving the use of *in vitro* models for better understanding GC-related mechanisms implicated in SRDs?

In **Chapter 6**, I zoom out and overlook all *in vitro* studies investigating the neurobiology of GCs (including our own), and question what challenges exist in the field, and what future considerations are required to move the field forward. Some of the challenges include (i) conceptual ambiguity of stress parameters *in vitro*, (ii) variability in cell models and cell types, (iii) variability in protocols, and (iv) variability in assessing glucocorticoid-induced phenotypes *in vitro*.

The lack of replication among studies (particularly those reported in **Chapter 2**) is concerning but not entirely unexpected given several sources of variability. The most striking difference between the studies involves the great range of GC concentrations used, with concentrations ranging from as low as 1nM to as high as 2.5 mM. Additionally, GC exposure paradigms vary greatly between *in vitro* studies, and the modeling of acute or chronic exposure is unclear in many of the listed studies. Although these studies do provide supporting evidence for several *in vivo* studies regarding the effects of stress and GCs on neurobiological mechanisms and pathways, improved standardization of GC paradigms *in vitro* is essential for improved replication and translational purposes.

The current literature on the neurobiology of GCs suggests a lack of standardization in the field, which creates a barrier for replication and impedes our understanding of the underlying mechanisms of GC-related stress. Therefore, I call on researchers to adopt a systematic and scientific approach in addressing these challenges. To this end, I propose a few recommendations to guide future *in vitro* GC studies. Firstly, I suggest the need for a clear definition and differentiation between acute and chronic stress *in vitro*, as well as their associated parameters. This could facilitate the modeling of specific stress mechanisms including allostasis and allostatic load *in vitro*. Pure cell culture (neuronal or glia) studies are crucial for disentangling cell-specific mechanisms in response to GCs and can deepen our understanding of the role of specific cell subtypes in stress susceptibility mechanisms implicated in SRDs such as PTSD. Moving away from using unreliable cell lines (such as immortalized neuroblastoma lines) and more towards improved cell models (including PSC lines) is recommended, however I do acknowledge that advanced stem cell models are similarly characterized by different sources of variation[9]. Applying a combination of readouts (particularly neuronal-specific readouts) should be used to assess GC-induced cytotoxicity, viability, and GC-induced phenotypes. These might include assessment of neuronal morphology, expression of GC-responsive genes (e.g.,

FKBP5, and *SGK1*), and transcriptional associated hallmarks, among others. Finally, focusing on a set range of GC concentrations (within 1nM and 100 µM) may be one step in reducing conflicting results.

Setting predefined criteria for *in vitro* studies investigating the neurobiological effects of GCs could lead to developing standardized and reproducible studies aimed at understanding underlying mechanisms of SRDs.

Research question 5: What are possible ethical implications in the use of advanced human stem cell models for stress-related research and identification of biomarkers for PTSD susceptibility?

While '*Stress-in-a-dish*' may sound like a catchy title for an *in vitro* stress model, I realize that our approach is simplistic and that any findings should be interpreted very cautiously. In fact, I am actually using it to spark a conversation about the ethical implications of studying stress and SRDs. We attempt to showcase that *in vitro* studies are not devoid of ethical implications as one might assume, but instead may carry ethical implications associated with increased complexity of the models used in the case of cerebral organoids and can even facilitate the development and implementation of technologies that are ethically loaded, in the case of biomarkers for SRDs. In **Chapter 7** I argue that when discussing the ethics of research on cerebral organoids, it may be misleading to assume that all organoids have the potential for sentience. Instead, it is recommended to distinguish between guided and unguided organoids for ethical analysis. To avoid unnecessary ethical oversight, future research ethics framework must determine what to protect: (i) complex brain models like assembloids, (ii) unguided cerebral organoids with a composition similar to the developing brain, and/or (iii) guided brain organoids. Moreover, the use of cerebral organoids is justified as an improved model to answer specific research questions and not to replace all other *in vitro* and *in vivo* models. That being said, while oversight is not necessary at this moment, limiting their use in non-essential research, especially stress-related research, may avoid potential ethical issues in the future. The media may also influence the informed consent process regarding the use of cerebral organoids for stress-related research. The use of sensationalized and exaggerated claims of what cerebral organoids are, including their uses and potential implications, may mislead non-experts and particularly potential donors. It is important to exercise caution in communicating this research to the public and potential donors, countering the impression that it may cause harm to 'sentient' organoids. Therefore, guidelines for

ethical communication of cerebral organoid research should be developed for scientists, journalists, and science communicators to prevent false hope and hype. This can improve the public's understanding of cerebral organoids without misrepresenting their potential. Finally, using chimeras (i.e., combination of cerebral organoids and rodents, with cerebral organoids being transplanted in rodent brains) in stress research is a double-edged sword that must be carefully considered. While it may increase the validity of animal models and address criticisms of translational potential, it may also lead to reduced welfare of the animal and induce more pronounced psychiatric-like symptoms.

Chapter 8 highlights the ethical, legal, and societal implications of identifying biomarkers for PTSD susceptibility in the context of military and law enforcement members. It has been postulated that increased paternalism together with decreased autonomy may overrule the act of beneficence in introducing and applying this technology. Moreover, one could argue that this technology could increase stigma and discrimination within military or police members for example. On another note, the identification of PTSD susceptible individuals may also go hand in hand with identifying PTSD resilient individuals which calls for careful consideration of how this may influence dual-use practices and carry implications for moral injury. The ethical, legal, and societal relevance of the research described in this thesis will be discussed in the impact chapter (**Chapter 10**) of this thesis.

Strengths and limitations

Several strengths of the studies presented in this thesis are worth mentioning. For instance, **Chapter 3** is novel in presenting a robust paradigm required to induce a chronic CORT-induced phenotype in human cortical neurons at different stages of neuronal development. Additionally, **Chapter 4** presents a unique approach to investigating chronic CORT effects on neuronal processes, by making use of trajectory inference analysis to highlight genes driving neuronal differentiation, followed by the identification of CORT-sensitive genes throughout stages of neuronal differentiation. By applying this CORT paradigm, the experiments performed in **Chapter 5** are among the first to assess CORT-responsiveness of candidate genes associated with SRDs, namely military combat-related PTSD, and the first to assess their role and relevance in stress susceptibility-related mechanisms. Another common strength in these studies is the use of a translational *in vitro* model using stem cell technology, specifically hESC-derived cortical neurons. Given their human origin, this model allowed the demonstration of human-specific cellular responses. Moreover, their use facilitated the investigation of neuronal subtype-specific responses to CORT.

Chapter 7 focused on the ethical implications of *in vitro* studies using advanced models in stress-related research. Although there has been increasing literature on the ethics of cerebral organoid use, here I provide novel insights requiring increased consideration in future research on cerebral organoids for stress-related research. In **Chapter 8**, I covered the future ethical implications of the identification of biomarkers for PTSD susceptibility. While the ethics of biomarkers for psychiatric disorders have been subject of extensive debate, I take a proactive approach in highlighting some of the foreseen implications that will emerge with the implementation of the technology.

This brings us to the main limitations of the studies presented in this thesis. The most critical might be the small sample size of the cell culture studies. While most of the currently observed effects in these preliminary studies seem quite robust, the power of the studies is limited which may influence the reliability and generalizability of the findings. This now requires further validation and replication with increased sample size experiments.

Regarding the chronic CORT paradigm, several things need to be pointed out. First, the use of CORT only to mimic aspects of stress mechanisms has been long criticized for being reductionist and not capturing the complexity of underlying stress mechanisms. Although this does not undermine the importance of investigating the effects of individual stress hormones and molecules, further investigation of the neurobiological effects of stress hormones in combination are highly warranted. Moreover, the CORT concentration used (100 *μM)* in our *in vitro* experiments are likely much higher than physiological concentrations in the human brain following chronic stress. In serum for example, baseline CORT concentrations range between 137nM and 283nM[10], whereas pathological states are associated with higher levels in the 420-779nM range[11]. There are several reasons why this high concentration could be justifiable in vitro, including binding of GCs to the culture dish, availability of GCs in the culture medium, among others[4]. This is not to mention the difficulty of measuring accurately physiological concentrations in humans, even in situations where it is easier to do so (e.g., maternal stress and pregnancy[1]). Although the use of high GC concentrations may be justifiable *in vitro*, the lack of consensus on what constitutes a standardized concentration range is worrisome[12]. This calls for standardization practices regarding GC concentrations, exposure times, and readout parameters among others, for *in vitro* studies investigating GC (and other stress hormones). This will allow improved reproducibility of results and increased validity of the model.

A limitation of our candidate gene experiments is the inclusion of only two candidate genes associated with PTSD susceptibility. It is unlikely that just one or two candidate genes will one day serve as robust biomarkers for identifying PTSD susceptible

individuals. Psychiatric disorders in general are highly complex and might require multidimensional approaches, including biological, psychological, and behavioral biomarkers[13, 14], and not only relying on DNA methylation differences, as in the case of ZFP57 and DUSP22. Nevertheless, some might even argue that focusing on other genes with increased evidence regarding their implication in SRDs (e.g., BDNF[15] and FKBP5[16]) might have been a preferable choice to test the efficacy of the chronic CORT exposure paradigm. However, the purpose of this study was to investigate novel candidate genes for PTSD susceptibility.

The studies investigating genome-wide effects in **Chapter 3** and **Chapter 4** could have benefited from qPCR validation of the top differentially expressed genes. Nevertheless, given the high specificity and sensitivity of the Illumina NovaSeq system, qPCR validations have been questioned as having added value[17], therefore I relied on the sequencing findings alone.

Finally, the cortical neurons generated in the studies are not composed of pure cortical neuronal cultures. During the differentiation process, NPCs are also capable of differentiating into glial cells[18], which can produce potential confounding factors. On the one hand, glia contamination is rather small, and it could be argued that the presence of glial cells may lead neuronal cultures to exhibit an improved *in vivo*like response. However, future studies aimed at disentangling the CORT effects on cortical neurons from possible confounders would benefit from generating pure cultures of cortical neurons. Moreover, while our model was able to capture a CORT-responsive phenotype, a recent study exploring different PSC differentiation methods has demonstrated that the transdifferentiation method might be an improved method over indirect conversion of PSCs, for generating improved responsiveness of neurons to GCs[19].

Future perspectives

The main findings of this thesis, together with the listed strengths and limitations bring about opportunities for future research into ELS and underlying stress mechanisms. On the one hand, the identified CORT-induced phenotypes and underlying molecular pathways in different stages of neuronal development, and the effects of CORT on PTSD candidate genes, provide a solid foundation for future functional studies exploring the underlying mechanisms of ELS. On the other hand, the limitations might in themselves be used to improve *in vitro* paradigms to investigate GC-related mechanisms in ELS and SRDs. The work in this thesis can be considered as a stepping stone onto which future studies can build.

Several lines of research are suggested here, that could move this field forward. As previously mentioned, the use of stem cell models brings about several opportunities for research in the field of stress. For instance, the use of iPSCs derived from patients suffering from SRDs might allow the modeling of disease-specific mechanisms[5], and improve our understanding of gene-environment interactions mediating stress susceptibility. Moreover, this technology allows the generation of unique neuronal subtypes, including serotonergic neurons, and glial cells, including microglia, that have been previously shown to be associated with SRDs, and hence could provide additional findings on the effects of GCs on different neurotransmitter systems. In addition, this facilitates looking into cell-cell interactions between cells derived from the same individual, by using co-culture techniques for investigating the effects of GCs on for example astrocyte exposed cells co-cultured with non-exposed cortical neurons. This would further highlight the effects of GC exposure on glia cells and how these impact their supportive functions. Importantly, stem cell-derived models (including 2D neurons resulting from indirect conversion methods and 3D cerebral organoids) remain one of the optimal *in vitro* models for modeling neurodevelopmental processes and disorders (including prenatal stress and ELS), and as such moving away from less robust models is strongly advised. While stem cell-derived models offer great advantages and opportunities, this technology comes with its own set of challenges[9] that should not be overlooked, including variability in differentiation protocols, between cell line variability, difficulties modeling complex brain diseases among others.

Another important challenge is the investigation of the effects of a combination of stress hormones (e.g., GCs and NA) in a way that accurately models acute or chronic stress mechanisms, and better reflects *in vivo* situations. In this thesis, I highlight the need for standardized methods to improve reproducibility of *in vitro* GC studies. This of course would be a pre-requisite to the investigation of two stress hormones or more, to avoid the same challenges.

To date, the precise role of PTSD candidate genes, DUSP22 and ZFP57, in the central nervous system is still unknown, and their biological relevance to susceptibility for SRDs is unclear. In order to bridge this knowledge gap and create supportive evidence of their putative relevance, both *in vitro* and *in silico* functional studies are required. While the former could include manipulation of the expression of these candidate genes and assess the effects on neuronal processes including neurogenesis and neuronal morphology among other outcomes, the latter may make use of multi-omics approaches to investigate genome-wide potential alterations in neuronal function. Explorative studies from iPSC-derived neurons from PTSD susceptible and resilient individuals could also add insight into the role and relevance of these genes in stress susceptibility mechanisms.

Alternatively, the chronic CORT paradigm could be used to investigate the effects of CORT on other SRD candidate genes as a way to provide evidence for their potential role in stress-related mechanisms.

Even though *in vitro* models (particularly iPSCs) offer increased validity, the use of animal models remains an important avenue to validate many of the novel *in vitro* findings. There remain challenges in translating basic science discoveries including *in vitro* studies, to clinical practice, and hence combining *in vitro* and *in vivo* studies where possible could be valuable in mending this gap.

Importantly, the ethical implications of advanced *in vitro* models, and the use of biomarkers for psychiatric disorders requires further research and ethical analysis. For instance, investigating how current science communication practices may influence the public's perception on cerebral organoid research in relation to stress is of great importance for providing best practices on how to ethically communicate sensitive research without contributing to hype or false hope. Additionally, investigating the potential implications of biomarker testing in certain professions (e.g., military and law enforcement) is crucial in preventing misuses of the technology.

To conclude, it is needless to say how vital *in vitro* research is to improve our understanding of biological effects environmental stressors, including ELS, and how these may contribute to SRDs or neurodevelopmental disorders. Advanced *in vitro* models create opportunities for improved modeling of neurodevelopment first, and gene-environment interactions second. More research is required to tackle the challenges and limitations of investigating individual stress hormones such as GCs, and to improve methodologies for modeling different aspects of stress. The studies presented in this thesis contribute to an understanding of the recurrent challenges in the field, and to mechanisms implicated in chronic stress throughout neurodevelopment. Together with other efforts, this work serves as one of the building blocks for further advancing our understanding of stress susceptibility and hopefully for new ways to alleviate the suffering of those with SRDs.

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9

Chapter 10

Summary

The aims of this thesis were (1) to develop an *in vitro* model of ELS (i) to investigate the neurobiological effects of chronic cortisol (CORT) in human cortical neurons throughout neuronal development and (ii) study candidate genes associated with PTSD susceptibility, and (2) to highlight the ethical implications that may result from this research. This thesis presents work done on the first aim in Chapter 2 to 6 while aim 2 was covered in Chapter 7 and 8.

This thesis starts with reviewing the available literature on *in vitro* studies investigating glucocorticoids (GCs), which was summarized in a review presented in **Chapter 2**. This overview highlights the different mechanisms and pathways affected by GC exposure in central nervous system cells and the need to standardize GC *in vitro* studies, starting with an extensive definition of stress *in vitro*. Future research ought to focus on how GCs play the role of mediators in different types of stress, cell type-specific vulnerabilities to GCs, GC-mediated mechanisms in stress susceptibility and resilience, the interplay between GCs and other stress-related hormones and explore ways to improve *in vitro* modeling of stress mechanisms.

Next several experimental studies were performed using human embryonic stem cell-derived cortical neurons at different stages of neuronal development. In **Chapter 3**, I investigated the effects of chronic CORT in neural progenitor cells (NPCs), immature young neurons, and maturing neurons. A decrease in proliferation and survival were observed in NPCs, a decrease in differentiation-related markers was observed in young immature neurons, and changes in synaptic plasticity proteins, neuronal activity, and glial marker in maturing neurons. Moreover, genome-wide changes were observed in the distinct stages of neural differentiation including dysregulation in synaptic plasticity, GC signaling, and extracellular matrix organization among other pathways. The findings of this study are supported by previous *in vitro* and *in vivo* studies showing negative effects of GCs on neuronal processes.

To gain better insight in the longitudinal effects of chronic CORT on neuronal development, trajectory modeling of transcriptomic patterns was performed. This yielded patterns of genes driving differentiation, from which subsequently CORT-sensitive genes were identified by analyzing whether their expression changes interacted with chronic CORT. The findings were presented in **Chapter 4** and highlight a set of genes involved in neuronal differentiation and synaptic plasticity as being sensitive to CORT-exposure. Together these findings identify genes and related processes dysregulated in GC-mediated mechanisms, in accordance with pathways previously shown to be implicated in ELS, thereby enhancing their potential implication in SRDs and validating the modeling of stress-related mechanisms *in vitro*.

In **Chapter 5**, I then applied this established chronic CORT ELS model to maturing cortical neurons to investigate candidate genes involved in epigenetic mechanisms underlying susceptibility to post-traumatic stress disorder (PTSD). This study is the first to assess the biological relevance of these genes in relation to stress mechanisms *in vitro*. The findings resulting from this exploratory study highlight the CORT-responsiveness of these genes through the observed changes in DNA methylation and mRNA expression of *DUSP22* and *ZFP57*, respectively. Moreover, this increases the relevance of these genes as potential biomarkers for PTSD. More research is needed to assess the functional relevance of these genes in neuronal processes and in relation to GC-associated mechanisms *in vitro* and *in vivo*.

An overview of the challenges and limitations of GC *in vitro* studies was presented in **Chapter 6**, together with future directions for the field. The existing sources of variability between these studies makes it increasingly challenging to have reproducible and conclusive results on the neurobiological effects of GCs. Although the existing literature exploring the effects of GCs is extensive, future studies should adhere to standardized practices to ensure reproducibility and increased validity of *in vitro* studies investigating neurobiological effects of GCs and related stress hormones. Table 1 summarizes our main findings from Part I of this thesis.

Table 1. A summary of the main findings in Chapter 3, 4, and 5 of this thesis.

	Neural Progenitor Cells (PRO)	Young Immature Neurons (Diffy)	Maturing Cortical Neurons (Diffm)
Chronic CORT-induced phenotype	o Decreased proliferation o Decreased survival o Increased apoptosis o Dysregulation in synaptic plasticity pathway	o No effect on overall differentiation o Decreased Pax6 expression o Dysregulation in GC signaling and extracellular matric organization	o Increased expression of post-synaptic plasticity protein o Age-dependent decreased neuronal activity o Decreased GFAP expression o Dysregulation in transmembrane receptor protein serine/threonine kinase signaling pathway
Chronic CORT effects on neurodevelopment	o Decreased LRRTM2 expression o Increased TSPAN5 expression o Dysregulation in synapse formation processes		o Decreased KCND3 expression o Decreased KCNIP4 expression o Decreased GRIA3 expression o Dysregulation in neuronal excitability and synaptic plasticity processes
Chronic CORT effects on PTSD susceptibility candidate gene methylation and expression	Not assessed	Not assessed	o Decreased DUSP22 DNA methylation in one CpG o Increased ZFP57 mRNA expression

Part II of this thesis dealt with the ethical implications, starting with **Chapter 7** which highlights the ethical implications of making use of human cerebral organoids (COs) in stress-related research. Ethical considerations pertaining to research ethics frameworks of *in vitro* studies, donor-related issues, and chimera research is discussed, while providing recommendations on how to navigate this uncharted area for future studies. Figure 1 summarizes our main conclusions.

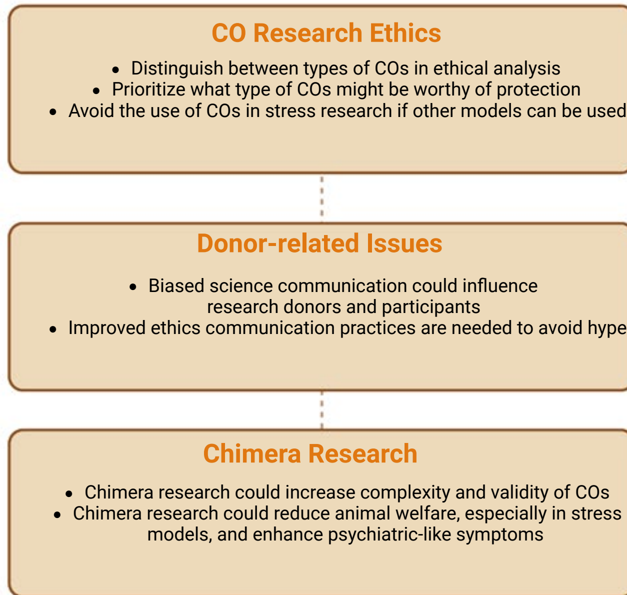

Figure 1. A summary of the main conclusions in Chapter 7.

Finally in **Chapter 8**, I highlight some of the ethical implications associated with the introduction of biomarkers of PTSD susceptibility and resilience in members of the military and law enforcement agencies. The identification of susceptible and resilient individuals brings about unique ethical considerations that require increased attention while the technology is still developing. This may bring about changes in policies that may carry consequences at the ethical, legal, and societal level. Figure 2 summarizes some of the main issues to attend to in the future.

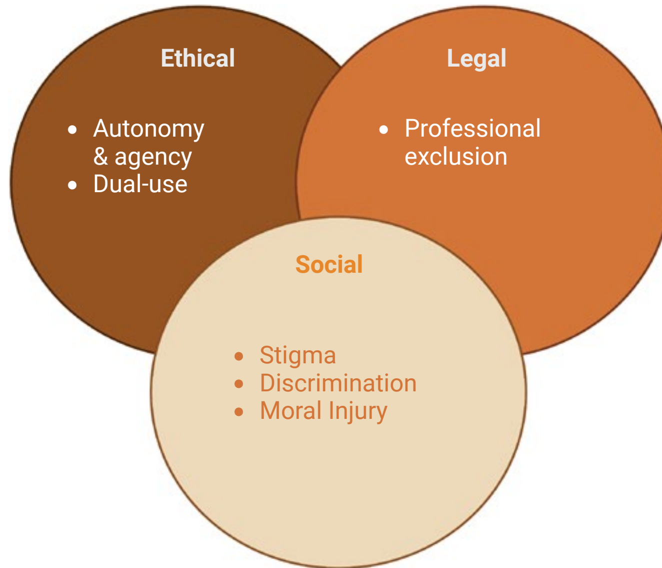

Figure 2. A summary of the main conclusions in Chapter 8.

Thus, the work described in this thesis provides novel insights into biological underpinnings associated with stress-related mechanisms and raises awareness about the ethical considerations that may arise from these pursuits. Nevertheless, it is important to acknowledge that this thesis contributes to a small portion of the larger efforts aimed at discovering the molecular and biological mechanisms underlying differential susceptibility to the effects of stress and SRDs, while also taking the lead in highlighting ethical considerations of stress-related *in vitro* research that require further attention and exploration.

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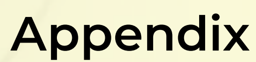

Appendix

Impact Paragraph
Curriculum Vitae
List of Publications
Acknowledgments

In this section, the scientific and societal impact of the research presented in this thesis will be discussed.

Scientific impact of glucocorticoid *in vitro* research

While there is an established connection between early life stress (ELS) and stress-related disorders (SRDs) in adulthood such as post-traumatic stress disorder (PTSD) and major depressive disorder (MDD), it remains unclear why some individuals are more susceptible to stress than others. Despite significant investments in understanding the etiology and pathophysiology of psychiatric disorders to develop new therapies, the mechanisms behind stress susceptibility and resilience remain elusive. Stress susceptibility and resilience have been associated with a number of genetic, epigenetic, and environmental risk factors, that together are thought to contribute to the development (or not) of SRDs[1]. However, to date, there are no robust and objective ways to identify susceptibility in at risk individuals, for example in deployed military members with a known high prevalence of PTSD. Thus, there remains unmet scientific needs to develop an ameliorated understanding of the molecular mechanisms underlying ELS and how they contribute to stress susceptibility and development of SRDs, which in turn could lead to the establishment of preventative interventions and diagnostic alternatives.

Research into the mechanisms of SRDs, such as MDD and PTSD, had improved our understanding of the underlying causes of these conditions, namely the role of excessive glucocorticoids (GCs) on the developing brain as a result of ELS. This research has provided insights into the negative effects of increased levels of cortisol (CORT) on a number of neuronal processes that are vital in ensuring healthy brain development, and on implicated pathways that lead to the development and maintenance of stress-related disorders as a consequence. Preclinical and clinical research has paved the way for advances in the field of research into ELS. However, there remains many questions that *in vivo* research with animals and humans cannot answer. Significant advances have been made in the starting materials (e.g., primary versus reprogrammed cell lines) and complexity of *in vitro* models being used to investigate GC-induced changes in neuronal cell types over the last few years. Stem cell technology allows us to investigate the effects of GCs on diverse neuronal subtypes with brain region specific phenotypes, in human-derived neurons. Today, it is possible to investigate the effects of a drug or chemical on cultured neurons generated from patient cells such as fibroblasts, or blood cells while preserving their genetic information. For instance, induced pluripotent stem cells (iPSCs) generated from patients suffering from PTSD can be differentiated in

different neuronal subtypes and compared to healthy controls to identify phenotypical differences *in vitro* that can be characteristic of the disease. Similar attempts can help researchers identify cellular and molecular pathways that may be disrupted in SRDs and potentially lead to the development of new targeted treatments. Alternatively, while more challenging for complex mental disorders such as SRDs, iPSC technology in combination with gene editing tools such as CRISPR/Cas9 could be used to shed light on the role of specific genetic variants and epigenetic modifications on the underlying SRD-related phenotypes. However, for these goals to be met, robust and standardized methods, large and well-characterized cohorts, and rigorous ethical guidelines must be established.

Abnormalities and altered functioning in the prefrontal cortex of patients suffering from MDD and PTSD have been reported[2], however, to date it is yet unclear how the effects of ELS contribute to the anomalies observed in cortical neurons. So far, research has not yet focused on the effects of GCs in human cortical neurons at different stages of neuronal development. Healthy maturation of the prefrontal cortex is essential for the development of several important cognitive functions, including decision-making and emotion regulation, which have been shown to be negatively affected in SRDs. The studies described in this thesis aimed to unravel the chronic effects of cortisol (CORT) in a human *in vitro* neuronal model throughout key stages of cortical neuronal development, and to employ it as a model for investigating candidate genes implicated in SRDs, and their interaction with CORT-induced modifications. In doing so, we provided a compilation of read-outs of chronic CORT exposure in cortical neurons at different neurodevelopmental stages, and how they can be used to investigate candidate genes involved in SRDs like PTSD. In this thesis, we have provided added evidence of the negative effects of chronic GC exposure on cortical neurons and implicated mechanisms in SRDs. Although GCs are not the only hormones involved in the stress response and in conferring risk to SRDs, there is increased evidence backing up their role in the regulation of processes vital for neuronal development and functioning.

An overview of studies to date, exploring the use of *in vitro* models for investigating effects of GCs, is presented in **Chapter 2**, which includes but is not limited to human pluripotent stem cell-based studies. Furthermore, advantages, challenges, and considerations related to the use of different *in vitro* models for GC-related stress research is addressed. Despite the need for further standardization of GC exposure paradigms, and validation of stem cell based-models, overall, the promise of advanced stem cell-based *in vitro* models will improve our understanding of many disease mechanisms and revolutionize approaches for the testing of drugs (i.e., GCs and other stress hormones), and ultimately the identification of therapeutic targets for SRDs. For instance, improved

definitions of *in vitro* stress paradigms, the use of robust and increasingly complex models, implementation of strategies to reduce variation in protocols and GC-induced phenotypes assessment, which are discussed in **Chapter 6,** are anticipated to overcome lack of reproducibility between studies, and inconsistencies with *in vivo* studies, emphasizing their scientific impact. Taking steps to standardize similar *in vitro* studies will help researchers in the field to conduct studies that are reproducible and reliable in translating *in vivo* conditions and clinical settings. Furthermore, a standardized approach may also help researchers identify potential pitfalls and inconsistencies in existing experimental protocols. Eventually, more consistent findings from *in vitro* studies can facilitate reliable identification and investigation of emerging targets for diagnosis and treatment of SRDs. Moreover, the use of robust and reliable *in vitro* models is expected to contribute to animal welfare, by reducing the number of animal experiments.

The research described in the first part of this thesis builds upon this notion and highlights the scientific impact of the presented studies. More specifically, **Chapter 3**, identifies distinct chronic CORT-induced phenotypes of human embryonic stem cell (hESC) cortical cells, at three critical stages of neuronal development, i.e., neural progenitor cells, young immature neurons, and differentiated maturing neurons. Additionally, **chapter 4** describes an approach for trajectory analysis identifying genes driving neural differentiation through the three stages, followed by a subset of genes impacted by the effects of chronic CORT. To date there has not been an *in vitro* study investigating the cellular, molecular, and transcriptional alterations in human cortical neurons at different stages of development following exposure to chronic CORT. The findings can be useful in better understanding the consequences of chronic CORT exposure during early life on the development and maturation of the prefrontal cortex in humans, and future risk for SRDs. Particularly, the identification of stage-specific CORT sensitivities, and CORT-sensitive genes could be useful for researchers in the fields of neuroscience, psychology, psychiatry, clinicians and for mental health professionals to inform novel research regarding the impact of environmental stressors on the developing brain, the development of new and more effective treatments and interventions for individuals exposed to ELS and/or suffering from SRDs. Moreover, this research can have implications for a wide range of neurological and psychiatric disorders beyond SRDs, including neurodevelopmental disorders such as schizophrenia, as well as neurodegenerative diseases like Alzheimer's disease, with environmental stressors as a risk factor.

Even though this study makes use of hESCs as a source for neural differentiation, the availability of induced pluripotent stem cells (iPSCs) allows for the generation of patient-specific cultures leading to opportunities for future SRDs studies. In this regard, iPSCs derived neuronal cultures can be used to model gene-environment interactions in order to investigate unique disease- or healthy-specific responses to a GC challenge. This would

facilitate advanced studies on the cellular and molecular responses to a GC challenge, in PTSD susceptibility and resilience for example, an impactful step towards personalized medicine. The identified altered pathways and genes presented here following chronic CORT, could serve as a foundation for future patient group-specific *in vitro* studies in stress susceptibility. And the use of a similar GC exposure paradigm can be employed as a GC model for future *in vitro* research, in the field of stress-related disorders, to investigate candidate genes and their interaction with GC-associated signaling pathways. All in all, the GC paradigm in combination with the use of a stem cell model offers ample opportunities to *in vitro* studies into GC-related mechanisms in stress-related research.

Chapter 5 describes a proof-of-concept approach on the use of the GC exposure paradigm presented in **chapter 3**, as an *in vitro* model to investigate effects of chronic CORT exposure on candidate genes associated with PTSD susceptibility in a Dutch military cohort[3], with the ultimate aim to improve the understanding of their role in neuronal processes eventually leading to conferring stress susceptibility. A better understanding of mechanisms implicated in stress susceptibility and SRDs, through *in vitro* studies looking into the effects of GCs, is a closer step towards identifying novel robust biomarkers for early prediction, prevention, and personalized therapies for the treatment of symptoms. Identifying biomarkers for susceptibility or resilience to SRDs would be useful for a variety of individuals, especially individuals at increased risk of being exposed to stress and trauma, such as military personnel and members of law enforcement agencies. This would involve translating findings from *in vitro* studies to *in vivo* and clinical setting eventually developing diagnostic tools for improved prediction, prevention, and personalized therapies. Additionally, this will facilitate the development of preventative strategies and interventions to mitigate risk of SRDs, and influence education and outreach efforts to reduce stigma surrounding mental health, though the promotion of evidence-based interventions.

Anticipated societal impact

ELS has been associated with increased risk of developing SRDs in adulthood, physical health problems, and social problems including increased risk for substance abuse, and criminal behavior. For example, 45% of veterans with PTSD have experienced physical abuse during their childhood[4]. This does not only impact the individual that has experienced ELS, but their family, community, and society as a whole. ELS can have a wide range of impacts on society, at the economic, social, and public health levels. For example, the economic costs associated with childhood maltreatment across Europe is estimated at tens of billions of euros[5]. Everyone is at risk of experiencing stress early in life, however

certain risk factors for ELS include socioeconomic status, family dysfunction, parental mental health, parental separation or divorce, lack of social support, discrimination and marginalization, and community violence, and recent evidence points towards the transgenerational effects of early life stress exposure on the mental health of future generations. Therefore, there is an increased incentive and need to better understand mechanisms implicated in stress susceptibility during early stages of neuronal development that lead to the development of debilitating psychiatric disorders later in life, which this thesis attempts to explore. Understanding stress susceptibility mechanisms could lead to the identification of biomarkers for predicting susceptibility to SRDs, and hence moving towards strategies to alleviate the suffering of millions.

It is important to acknowledge that the research described in this thesis, although carrying scientific impact, remains fundamental and is in its preliminary stages. That being said, the studies presented here are most likely not going to have a direct impact on society any time soon, and in particular to individuals at risk or suffering from stress-related disorders. As such this thesis would like to avoid contributing to false expectations or hope that may get lost in translation. However, what this thesis provides are new insights and opportunities for other scientists in the field. Thus, the findings in this thesis contribute to the wider scientific community and particularly has impact for other scientists attempting to investigate the effects of chronic stress and underlying biological mechanisms by using experimental systems such as cell culture models. It may furthermore provide a basis for validation experiments of findings from *in vivo* studies. It may be expected that the further development and use of these model systems will yield the identification of actionable biological targets, which can form a basis for novel interventions aiming to reduce the impact of chronic stress on health, which is an enormous challenge in modern society.

Some of the aforementioned endeavors and promises driving progress in the field of psychiatric disorders, namely the use of advanced *in vitro* stem cell models and the use of biomarkers for the identification of PTSD susceptible individuals, also carry ethical, societal, and legal implications that need to be considered.

The use of advanced stem cell-based models, namely 3-dimensional (3D) cerebral organoids for investigating stress mechanisms in SRDs, offer promising avenues for improved modeling of SRDs and drug testing, in addition to the development of therapies for these disorders. However, the use of cerebral organoids raises ethical considerations that relate to research ethics practices. One of the major concerns according to some experts is the potential for these organoids to become sentient. Although evidence suggests that it is unlikely that cerebral organoids will have the

capacity for consciousness anytime soon, the possibility of its occurrence raises ethical questions about how to treat such entities. For example, similar to animal research, introducing research ethics committees that aid in the reinforcement of set ethical guidelines has been recommended[6]. More eminent ethical issues include donor-related concerns and ethical considerations surrounding the generation of chimeras with human cerebral organoids and animals. **Chapter 7** discusses all of the aforementioned ethical implications in the context of cerebral organoids for investigating stress mechanisms and stress-related disorders. The potential benefits of making use of cerebral organoids for stress-related research must be weighed against the potential harms. Taking steps towards mitigating these risks while maximizing the benefits is warranted. Having these discussions (though outreach activities or debates) with scientists working with stem cells (including cerebral organoids), with science communicators, ethicists, policy makers, patient organizations, and the public is crucial in advancing our understanding of the ethical considerations. Furthermore, working on guidelines for ethical communication of findings associated with cerebral organoids is warranted for responsible dissemination of the science without facilitating false hope and hype.

As previously mentioned, the findings presented in this research can be used for the future investigation of biomarkers for susceptibility to SRDs which could serve as both objective preventative and diagnostic measurements. Identifying the role of biomarkers in conferring susceptibility to SRDs open avenues for targeted personalized approaches, in addition to the development of novel therapies. In practice, biomarker testing for PTSD susceptibility can serve a large population of individuals at high risk of developing PTSD, particularly law enforcement members. Biomarker testing can be used in combination with current established mental health screenings during recruitment, pre- and post-deployment. This has the potential to reduce the occurrence of PTSD among law enforcement members, reducing the associated health, economic, and social burden that accompany PTSD. However, although research into psychiatric biomarkers is on the rise, the promise of psychiatric biomarkers has overlooked the ethical implications that require much needed attention[7]. **Chapter 8** describes some of the unique ethical, legal and societal considerations of introducing biomarker testing for PTSD susceptibility in the context of law enforcement, including increased stigma, discrimination, professional and social exclusion. The need for ethical analysis and research into biomarkers for PTSD susceptibility, particularly in the context of law enforcement agencies, is called for as this may also involve the identification of PTSD resilient individuals, hence the possibility for dual-use applications. Bioethical reflections, public engagement efforts, and inter-disciplinary collaboration are highly required to move the discussion forward before the technology is brought to the bedside.

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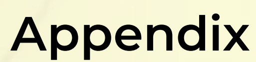
A1



Appendix

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Curriculum Vitae

Katherine Bassil was born on June 8th, 1995, in Ohio, the United States of America, and grew up in Lebanon, in the Middle East. She holds a bachelor's in Biology and a minor in Psychology from the Lebanese American University, where she graduated with *honors* in 2013. Her interest in the intersection of biology and psychology led her to pursue a Research Masters in cognitive and clinical neuroscience at Maastricht University, The Netherlands, where she specialized in fundamental neuroscience. During her studies, Katherine joined the lab of Prof.dr. Bart Rutten at Maastricht University and was introduced to *in vitro* models for the study of stress-related disorders, namely post-traumatic stress disorder. For her internship, Katherine took lead in setting up a project due to ongoing collaborations with the Salk Institute for Biological Studies, where she joined the lab of prominent neuroscientist Prof.dr. Fred H. Gage and worked under the supervision of dr. Krishna Vadodaria to complete her internship. She graduated in 2018 and received a PhD position to establish the differentiation of stem cells into neuronal subtypes at Maastricht University in the lab of prof. Rutten, under the supervision of dr. Laurence de Nijs, and dr. Gunter Kenis. During her PhD, Katherine worked on the development of an *in vitro* stem cell-based model to investigate the neurobiological effects of glucocorticoids on cellular and molecular processes, and potential epigenetic biomarkers. In parallel, Katherine dedicated significant time throughout her PhD to investigate the ethical, legal, and societal implications of this line of research with the help of dr. Dorothee Horstkötter.

In addition to her research experience, Katherine engaged in outreach and science communication activities including the organization of the first Neuroethics Symposium for students during her Masters; she initiated and led the Neuroethics Café, a series of meetings within Maastricht University highlighting discussions on the ethical, legal, and societal implications of neuroscience and neurotechnology among students, researchers, and staff. Katherine has given several public talks at several events including the international Pint of Science and PechaKucha events, and has written columns on topics ranging from neuroscience, neuroethics, and research integrity for Nature, and Observant, among others. Her biggest outreach initiative has been the development of a neuroethics education and communication platform "*Neuroethics Today*" where she raises awareness on neuroethics topics to scientists and the public, while partnering with the International Neuroethics Society to amplify its mission.

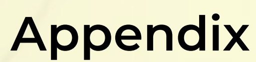
As part of her non-academic experiences, she has volunteered for different international neuroethics organizations at the forefront of ethics, data, and technology. For example, she has been a member of several committees for the International Neuroethics Society

(INS) since 2019, and since recently is the ethics lead of the IEEE Wellness workgroup drafting ethical guidelines for Wellness neurotechnologies. She is also a consultant for a startup company, providing advice on the development of a decision-making tool for neurosurgeons.

Katherine has recently been accepted to a Harvard Neuroethics Fellowship and upon completion of her PhD, will continue her academic career as a postdoctoral researcher in neuroethics at the University Medical Center (UMC) Utrecht in the Netherlands focusing on the ethics of (bio)medical innovation, in collaboration with the Technical University (TU) of Munich in Germany, in the Laboratory of Ethics of Artificial Intelligence and Neurosciences. **Figure 1** represents a graphical overview of her CV.

Figure 1. Overview of education and career trajectory (2013-2023)

A2



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Scientific publications

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Bassil, K. The end of 'mini-brains'! Responsible Communication of Cerebral Organoid Research. Invitation to contribute an opinion article for the special issue '*The Ethics of Brain Organoids*' in *Molecular Psychology*.

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While a thesis has one author, there are several actors, behind the scenes, that are involved in making it come to life. The support I received, be it emotional or work-related has been overwhelming and I am grateful for all those that have left a mark. I am not familiar with any other education or job that runs you through all sorts of emotions and experiences in just a few years, leaving you with lessons and friendships like a PhD does. Doing a PhD is by far one of the most challenging yet fulfilling experiences I have known. Doing a PhD is becoming a better scientist, but most importantly it is learning about yourself and others. It is a non-ending chain of giving and receiving. It is loving science and then hating it, and then loving it again. But throughout it all, you are never alone.

Having said that, there are several people that I would like to express thanks and gratitude towards. First and foremost, I would like to start thanking and acknowledging my promotion team, who made it possible for me to work with them during my Masters and PhD, and who gave me the independence and trust that any PhD could dream of.

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Gunter, we could agree that my PhD was not straightforward, it sometimes felt we were re-inventing the wheel, a task I often found frustrating. But throughout it all, you remained calm, focused, and supportive. One could say that is exactly what I needed, given how restless and impatient I can be sometimes. Your knowledge and insights in every meeting always left me puzzled, which made me realise that learning has no finish or end but is a constant and never-ending process. Work aside, your experiences with fatherhood have been a great source of reassurance, especially as I prepare for the journey myself. Hartelijk Dank!

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Daniel, there is a lot one can say about how influential you have been to me, and others like me. You stood up for me, you have constantly encouraged me and others to use their voice and speak up. You have taken on the task to raise awareness on issues related to social safety within academia, and throughout it all, you always remained hopeful and positive. And even when we disagreed, you have never treated me as inferior, but to the

contrary. It is unfortunate that it takes a senior to speak up for others to pay attention, but I am grateful that you did. Thank you for being a true EPIC leader, for all the engaging discussions, and for always being kind and thoughtful towards others. Besides that, you were always ready to provide me feedback and advice on my project, which was exactly what I needed, especially in times where I felt lost and needed an objective opinion. I am sure your spirit and values will catch on! Also, thanks to you, I will always be remembered as the Carpe(t) Diem PhD. Hartelijk Dank!

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Ghazi, thank you for all the delicious sweets, especially those you brought with you from Saudi Arabia. I wish you all the best! شكرا

For the second half of my PhD, I moved to room 1.156 with Phillippos, Rick, Sarah, and Valentin. Chris warned me that I would need an extension if I were to move there (Chris, I am sorry, but you were wrong). When the time came for me to write my thesis, I wanted to setup a home office and do the writing there. But I never did that. Instead, I wrote my whole thesis in a few months in this exact office I was warned to avoid due to increased risk of procrastination and waste of time (and I should add: bad influence). Looking back, I can honestly say, there is no way I would have written my thesis the way I did if it wasn't for this office. You all kept me accountable; you kept me sane, you were supportive, you lifted me up when I was about to hit rock bottom. You even (occasionally) acknowledged that I was funny. You were amazing! Thank You all.

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Philippos. Phillippos, Phillippos, Phillippos. If Sarah was a gift, you were a test. A test to m**y** debate skills, my patience, my nerves, my trigger p**o**ints, my weaknesses, my anger iss**u**es, my genuine funny natu**r**e, you na**m**e it! But the **o**ffice would have not been the sa**m**e without you. Thank you for regularly checking in on me when I was not having the best days, thank you for going against your will and being kind to me from time to time (Jk), and thank you for being supportive and funtastic. As for the trophy, you may keep it, I am taking the crown. Ευχαριστώ πολύ

Renzo, 'can I ask you a question?' I have probably approached you with this (and other follow-up questions) a few hundred times in the last couple of years, to get your advice and opinion on science and experiments. You have always been there to assist, help, and teach me new methodologies. You are one of the most brilliant minds I know, and I am positive we will soon call you professor Riemens. If scientific excellency had a description, it would most probably have your qualities. Kudos! Your passion for science is contagious. Hartelijk Dank!

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To future PhD graduates, never be afraid to speak up. Your voice is important, your opinion matters, and always remember your h-index does not define who you are. A PhD is not meant to be experienced alone, so do not be afraid to ask for help, lots of it! If you see a gap or inequities in the system, call them out! We are the change, and nobody is going to do it for us.

Last but not least, to those that said I could not do it, to those that called me a “scientist in theory” thank you! Thank you for showing me that naysayers do not and should not affect my dedication and my goals. Thank you for showing me that your beliefs of me do not define me. Thank you for showing me that yes, I can do it on my own, with a child, and far from home. Thank you for showing me that believing in myself outweighs all negativity and attempts to undermine my abilities. I did it! I did it, thanks to me and thanks to all those I was fortunate enough to have on my path. Someone on the internet once said:

*“There will be haters, there will be doubters, there will be non-believers, and then there will be **you**, proving them wrong.”*