Stress-in-a-dish

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 interdisciplinary collaboration are highly required to move the discussion forward before the technology is brought to the bedside.

References

1. Yehuda, R., *Biological factors associated with susceptibility to posttraumatic stress disorder.* The Canadian Journal of Psychiatry, 1999. **44**(1): p. 34-39.
2. Kroes, M.C., et al., *Structural brain abnormalities common to posttraumatic stress disorder and depression.* Journal of Psychiatry and Neuroscience, 2011. **36**(4): p. 256-265.
3. Rutten, B.P., et al., *Longitudinal analyses of the DNA methylome in deployed military servicemen identify susceptibility loci for post-traumatic stress disorder.* Molecular psychiatry, 2018. **23**(5): p. 1145-1156.
4. Zaidi, L.Y. and D.W. Foy, *Childhood abuse experiences and combat-related PTSD.* Journal of Traumatic Stress, 1994. **7**(1): p. 33-42.
5. Sethi, D., et al., *European report on preventing child maltreatment.* 2013: World Health Organization. Regional Office for Europe.
6. Hyun, I., J. Scharf-Deering, and J.E. Lunshof, *Ethical issues related to brain organoid research.* Brain research, 2020. **1732**: p. 146653.
7. Singh, I. and N. Rose, *Biomarkers in psychiatry.* Nature, 2009. **460**(7252): p. 202-207.