The hypothalamic – pituitary – adrenal axis and stress: An assessment of stress in a rat cell line and in a human cohort

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Ingunn,

Oslo, May 2021

Preface

Previous in-house social stress experiments in Sprague Dawley rats as well as analyses of human data from the general Norwegian population show that stress may have many effects on the hypothalamic-adrenal-pituitary axis. The present thesis is a continuation of work previously conducted by former and present members of Professor Johannes Gjerstad research group at STAMI.

In the *in vitro* arm of this thesis, I used data from the animal experiments mentioned above as a background for the cell culture experiments. Our lab engineer Anne-Mari Gjestvang Moe did the preliminary work (subcultured five times) with the RC-4B/C cell line (from ATCC® Company) that was used for the protein assay and RT-qPCR analyses.

In the human arm of this thesis, I used genomic DNA previously isolated from saliva provided by the human cohort. The DNA was isolated by PhD student Daniel Pitz-Jacobsen and lab engineer Aqsa Mahmood. For the statistical analyses I used data from a questionnaire that was distributed to the subjects (human cohort). The data from the questionnaire was processed by Anne-Mari Gjestvang Moe and Daniel Pitz-Jacobsen.

Excluding the experiments above, I have conducted all experiments, statistics, and analyses in this master's thesis. The experimental work includes cell culture exposure experiments, RNA and DNA isolation, immunoassay, synthesis of complementary DNA (cDNA), Real-time quantitative polymerase chain reaction (RT-qPCR) and single nucleotide polymorphism (SNP) genotyping.

Abstract

Previous research has shown that social stress can lead to various adverse effects, such as depression and anxiety. In the present thesis, the effect of stress was assessed *in vitro* with a rat cell line and with genotyping of a human cohort.

First, *in vitro* investigation of the stress response was conducted in a cell line from a rat pituitary adenoma, RC-4B/C. The stress response was triggered by stress hormones corticotropin-releasing hormone (CRH) and Dexamethasone (Dex) to examine the stress-induced changes in adrenocorticotropic hormone (ACTH) release (immunoassay), and on the expression of *Nrcam*, *Phf2* and *Skp1* (RT-qPCR). No clear effects of CRH or Dex were demonstrated in the cell line experiments.

Second, investigation of the consequence of social stress, in the form of exposure to workplace bullying, was assessed in human cohort consisting of 1226 Norwegian employees. The Hopkins Symptom Checklist and genotyping (SNP TaqMan assay) were used to examine the association between workplace bullying and symptoms of depression moderated by the CRHR1 haplotype (rs7209436, rs110402, rs242924). The result showed a possible correlation between female carriers of the CGG/CGG haplotype and a decreased effect of bullying in the workplace. Thus, the CGG/CGG haplotype may have a protective effect in females.

The *in vitro* arm of the present study suggests that the RC-4B/C cell line is not a good model for assessment of the CRH response in pituitary corticotroph cells. However, the human arm of the present study showed a clear association between social stress and symptoms of depression moderated by the *CRHR1* gene CGG/CGG haplotype.

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Abbreviations

ACTH	Adrenocorticotropic hormone
AVP	Arginine vasopressin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CORT	Cortisol (humans) / corticosterone (rats)
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin releasing hormone receptor 1
CTQ	Childhood trauma questionnaire
Dex	Dexamethasone
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Glucocorticoid
GH	Growth hormone
GPCR	G protein-coupled receptor
Nr3C1	Glucocorticoid Receptor
HSCL	Hopkins symptom check list
HPA	Hypothalamic-pituitary-adrenal
HWE	Hardy-Weinberg equilibrium
IV	Intravenous
JMJC	Jumonji C
LD	Linkage disequilibrium
LDH	Lactate dehydrogenase hormone
LH	Luteinizing hormone
MC2R	Melanocortin type-2 receptors

MDD	Major depression disorder
MAPK	Mitogen-activated protein kinase
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NAQ-R	Negative acts questionnaire - Revised
NRCAM	Neuronal Cell Adhesion Molecule
PCR	Polymerase chain reaction
PHF2	Plant Homeodomain Finger Protein 2
PKA	Protein kinase A
POMC	Proopiomelanocortin
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
RT- qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
SCF	Skp1–Cullin1–F-box-protein
SEM	Standard error of mean
SKP1	S-phase kinase-associated protein 1
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system

1. Introduction

1.1 The background for the present thesis

Stress can be defined as any internal or external stimulus that evokes a biological response [1], and it can affect the organism in many ways. Stress can affect homeostatic processes, memory [2], the immune system [3, 1], and lead to sleep deprivation [4]. However, it is important to note that stress has an important function in alerting the organism to various threats, and that the subsequent instigation of stress responses drives the organisms adaptation to potentially fatal surroundings [5]. The brain stem receives input concerning various stressors that may disrupt homeostasis, and consequently activates the "fight or flight" response of the sympathetic branch of the autonomic nervous system, in addition to an activation of the hypothalamic-pituitary-adrenal (HPA) axis [4, 6]. The function of these two systems is to help the organism adapt to the stressor in order to restore homeostasis, a process known as allostasis [7, 6]. Allostasis is mediated by hormones secreted from the activation of the SNS and HPA axis, such as epinephrine and cortisol, respectively. [8].

Stress can be divided into two categories: acute and chronic. The distinction between the two is both the duration and the consequences of the stress. The duration of acute stress is minutes to hours, whereas chronic stress lasts for 30 days or more [9]. Acute stress triggers the sympathetic nervous system (SNS) and the HPA axis. However, the stress response following an acute stressor ceases when the threat is removed and the internal balance, the homeostasis, is restored [5]. Chronic stress, on the other hand, causes stress responses that are not terminated quickly but rather lead to prolonged stress responses which has been associated with various disorders such as Alzheimer's and Parkinson's disease [10], depressive disorder [11, 12], and coronary heart disease [13].

1.2 The Hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal axis is a neuroendocrine system that regulates the body's response to external and internal stressful stimuli to maintain homeostasis.

In accordance with Britannica et al. [14], homeostasis may be defined as:

> *"Any self-regulating process by which biological systems tend to maintain stability while adjusting to conditions that are optimal for survival. If homeostasis is successful, life continues; if unsuccessful, disaster or death ensues. The stability attained is actually a dynamic equilibrium, in which continuous change occurs yet relatively uniform conditions prevail."*

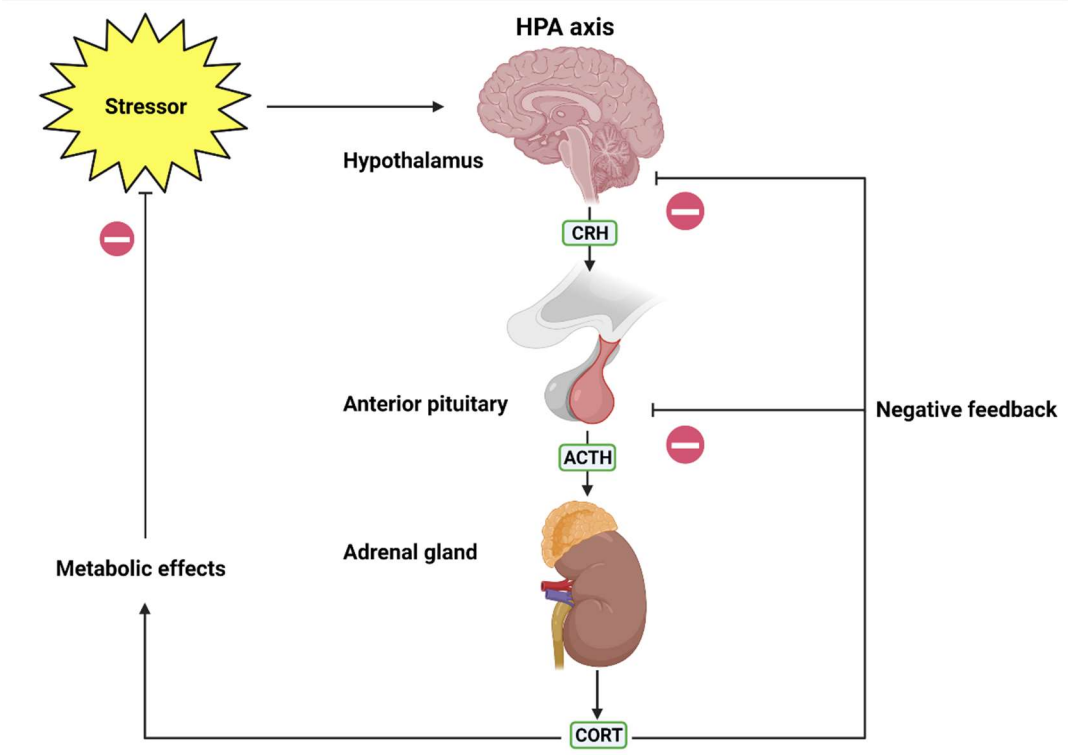


Figure 1.The HPA axis. This figure shows a schematic simplified overview of the HPA axis. Stressful stimuli lead to the subsequent hypothalamic release of CRH, which in turn leads to pituitary release of ACTH. Further, ACTH leads to synthesis and release of CORT from the adrenal glands. One of the main functions of CORT is to downregulate the HPA axis via a negative feedback process. This negative feedback prevents further release of CRH and ACTH from their respective organs. This figure was created with BioRender.com

The HPA axis consists of three components; the hypothalamus, the pituitary gland, and adrenal glands, which communicate through hormones [15] (Figure 1). The HPA axis is activated once a stimulus, e.g. an external or internal stressor, that may threaten the homeostasis in the body, is registered in the brain stem. The threat can be a real threat, e.g. excessive bleeding from a physical trauma, or a perceived threat, such as a social stressful situation. Both these situations may equally cause activation of the HPA axis. A perceived threat is also called an anticipatory response, because the HPA axis is activated in anticipation of an event that will disrupt homeostasis [16], i.e. a social stressful situation. As previously mentioned, a stressful stimulus will activate the SNS [11]. However, the SNS is not the focus in the present thesis.

As a response to stressors, the paraventricular nucleus (PVN) in the hypothalamus is activated and releases the pre-synthesized neurohormones corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into a portal blood vessel system [17, 15]. The PVN is a cluster of neurons that initiates the HPA axis cascade. This occurs after input of stressful stimuli from several brain areas, e.g. the limbic system, and the PVN responds in turn by secreting CRH. The limbic system consists of several structures, such as the amygdala and hippocampus. The system is largely involved in emotions and long-term memory, which connects to responses from psychological stressors [18].

CRH is a peptide hormone, stored in vesicles in the PVN, ready for immediate release upon a perceived threat to homeostasis. CRH is released into the portal blood vessels connected to the anterior pituitary gland, where it binds to corticotropin releasing hormone receptor 1 (CRHR1), a G-protein coupled receptor (GPCR), on corticotrophs in the anterior pituitary. Once CRH binds to CRHR1 receptors on corticotrophs, the hormone triggers the exocytosis of pre-synthesized adrenocorticotropic hormone (ACTH) and the synthesis of the ACTH precursor, proopiomelanocortin (POMC), to replenish the ACTH levels in the cell [15, 19].

Like CRH, ACTH is a peptide hormone. ACTH results from the cleavage of the prohormone POMC, which can be cleaved into a minimum of seven peptide hormones. Following cleavage, the resulting peptide hormones are sorted into vesicles that mature into secretory granules during transportation from the Trans-Golgi network to the plasma membrane, ready for secretion upon stimulation [20]. After CRH stimulates the release of ACTH from corticotrophs, the hormone follows the bloodstream until it reaches melanocortin type-2 receptors (MC2R) on the adrenal glands. The adrenal glands consist of a cortex and a medulla. The adrenal cortex is important in regulating homeostasis due to the production and secretion of glucocorticoids (GCs), the effector hormones of the HPA axis. The synthesis of GCs occurs in the cortex layer zona fasciculata. The medulla secretes epinephrine and norepinephrine as a consequence of SNS activation [21, 11]. Once ACTH reaches the MC2R receptors, the hormone executes its steroidogenic activity by initiating the process that synthesizes the GCs, cortisol/corticosterone (CORT), through the conversion of cholesterol [20, 15].

The hormone CORT differs from CRH and ACTH in its chemical properties. Unlike CRH and ACTH, CORT is a lipophilic hormone and cannot be stored in cells. Therefore, CORT is synthesized when required [19]. Cortisol or corticosterone is the main glucocorticoid hormone in humans and rats, respectively. Once synthesized, CORTs diffuse out of the adrenal glands and into the systemic circulation until they reach their targets and implement various metabolic effects. The targets are the two receptors for GCs, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) [19].

The effects of CORTs are various; the primary effects are on metabolism-, and the immune system, as well as a negative feedback effect to downregulate the HPA axis (Figure 1 and 2) [19]. The metabolic effects lead to the mobilization of energy required to correct the homeostatic imbalance. The energy is obtained by catabolic breakdown of glycogen, i.e. glycogenesis, in the liver, or by proteolysis and lipolysis [16]. In addition to obtaining energy via catabolic reactions, CORT also exerts various measures to prevent unnecessary energy usage, e.g. inhibition of bone- and muscle growth [16], and inhibition of the innate immune system [22]. Furthermore, CORTs are involved in the regulation of reproduction, behavior, and cognitive functions [19].

The elevated levels of CORT that follows HPA axis activation is utilized to promote adaptation to stressors in the organism and reestablish the homeostasis. Another important function of CORT is the downregulation of the HPA axis (Figure 1 and 2). This downregulation occurs through a negative feedback effect exerted by CORTs on the hypothalamus and the anterior pituitary gland, thus preventing further release of CRH and ACTH, respectively. Subsequently, the secretion of CORTs is downregulated [19].

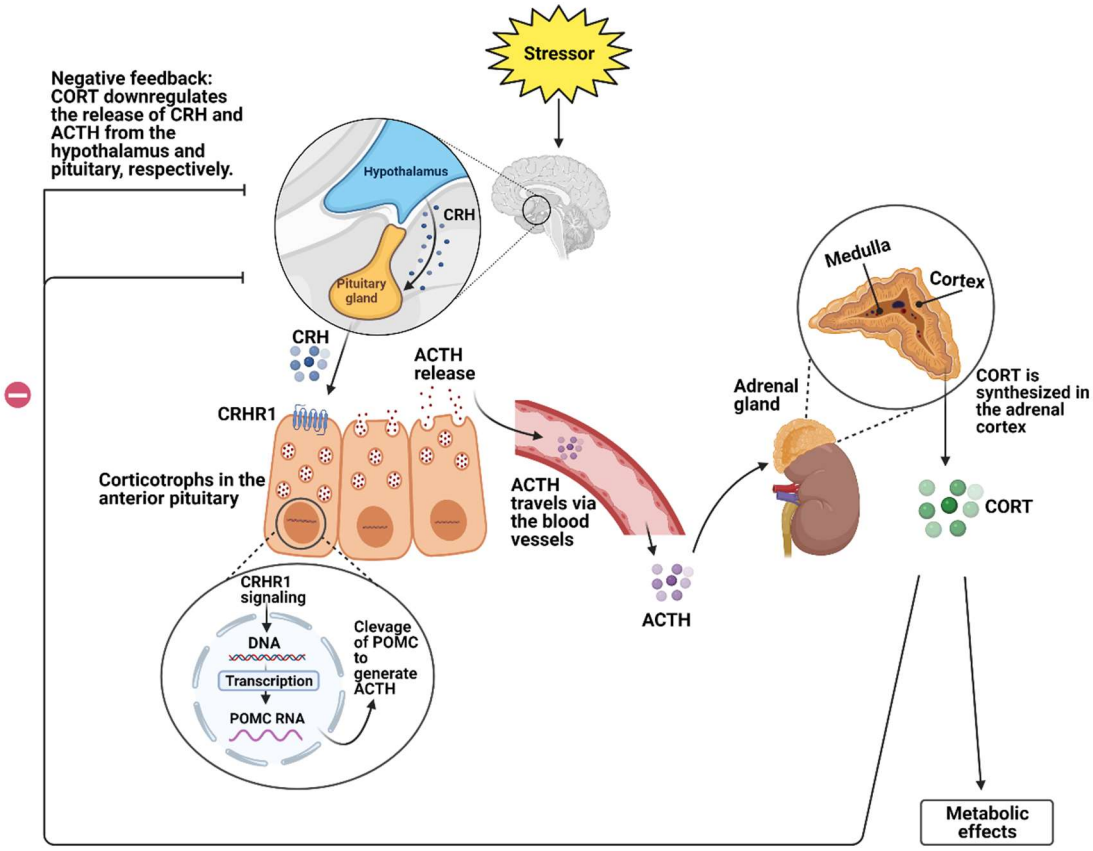


Figure 2. Schematic overview over the HPA axis. Stressful stimuli cause activation of the HPA axis with a subsequent release of CRH from the hypothalamus. CRH binds to CRHR1, GPCRs on corticotrophs in the anterior pituitary. The activation of CRHR1 triggers a downstream signaling pathway that ultimately leads to the transcription of the prohormone POMC and the cleavage of POMC into smaller peptides such as ACTH. ACTH is stored in secretory vesicles near the cell membrane and released when CRH binds to CRHR1. ACTH travels through the blood stream to the adrenal glands where it functions as a steroidogenic hormone; binding to receptors trigger the production and subsequent release of CORTs from the adrenal cortex. CORT elicits many metabolic effects for the organism to counteract the stressful stimuli. CORT also has a negative feedback effect; it downregulates the upstream release of CRH and ACTH, from the hypothalamus and pituitary, respectively. This figure was created with BioRender.com

In addition to the HPA axis' involvement in the stress response, the axis also plays an important role in basal activities of the body. The basal HPA axis activity is grouped into two rhythms, an ultradian and a circadian rhythm, and humans and rats both share these basal activities. The ultradian rhythm involves a secretion of CORT and ACTH that occur in pulses approximately every 60 minutes [23]. The circadian rhythm involves a daily fluctuation of CORTs with a peak at the start of the circadian active period. The time of the peak differs in humans and rats; humans have a peak basal level during the morning, whereas rats are nocturnal animals, and thus have their basal peak at the beginning of the evening [24].

1.2.1 The HPA axis and associated diseases

As previously described, the HPA axis is an essential system for the body to adapt to stressors to maintain homeostasis. However, if the stress response is not regulated normally but is excessive, prolonged, or inadequate, allostatic load occurs. Allostatic load describes a state in which the cost of reestablishing homoeostasis becomes too high, which can be caused by a hyper- or hypoactive HPA axis [7, 25, 18]. Furthermore, chronic stress can lead to elevated levels of CORTs due to the lack of downregulation of the HPA axis in the presence of a long-term or constant stressor. This hyperactivity of the HPA axis can also affect other biological systems, which may cause negative effects both psychologically and physically due to the elevated levels of CORTs. Previous research have found that consequences of such dysregulation is associated with psychiatric disorders, such as depression, posttraumatic stress disorder (PTSD) and chronic fatigue syndrome [26]. Many of these disorders occur in individuals who are genetically predisposed [27, 18].

However, not all diseases related to the HPA axis are caused by an abnormal stress response. Some diseases like Cushing's disease, which is due to excessive levels of CORTs [26], or Addison's disease, due to insufficient levels of CORTs, are caused by other factors [28].

1.3 The rat model and the RC-4B/C cell line

Rats, *Rattus norvegicus,* are frequently used as model systems in neuroscience, partly because the HPA axis is highly conserved in both humans and rats [29]. Rats can be used as model systems to study various stressors and the subsequent stress response. The resident-intruder paradigm is a well-established method to assess the effects of social defeat in rats in the form of behavioral, physiological and neurobiological effects [30]. This method can provide information that may translate to similar situations and responses in humans.

1.3.1 Results from our research group

Our research group has previously assessed social stress in Sprague Dawley rats with the resident-intruder paradigm [31, 32]. An overview of the process can be seen in figure 3, and the method itself is described in the method section 3.1. The results from previous studies from our research group for the exposed rats compared to the control rats were: decreased body weight, decreased expression of the *Pomc* gene in the pituitary gland, downregulation of the GC receptor Nr3C1 in the adrenal glands, and a significant increase in the expression of the GC receptor Nr3C1 in the pituitary gland.

The purpose of utilizing the resident-intruder model was to simulate a socially stressful situation in the Sprague Dawley rats. Information from this model can subsequently be used to find possible candidate genes that affect the stress response in humans. For instance, our research group has previously found a stress-induced upregulation of several microRNAs in the Sprague Dawley rats after the resident-intruder paradigm. From these findings, they were able to translate the results to humans via genotyping. The subsequent analyses in humans revealed that certain genotypes in some of the stress-upregulated microRNAs led to a stronger association between exposure to workplace bullying and pain [33].

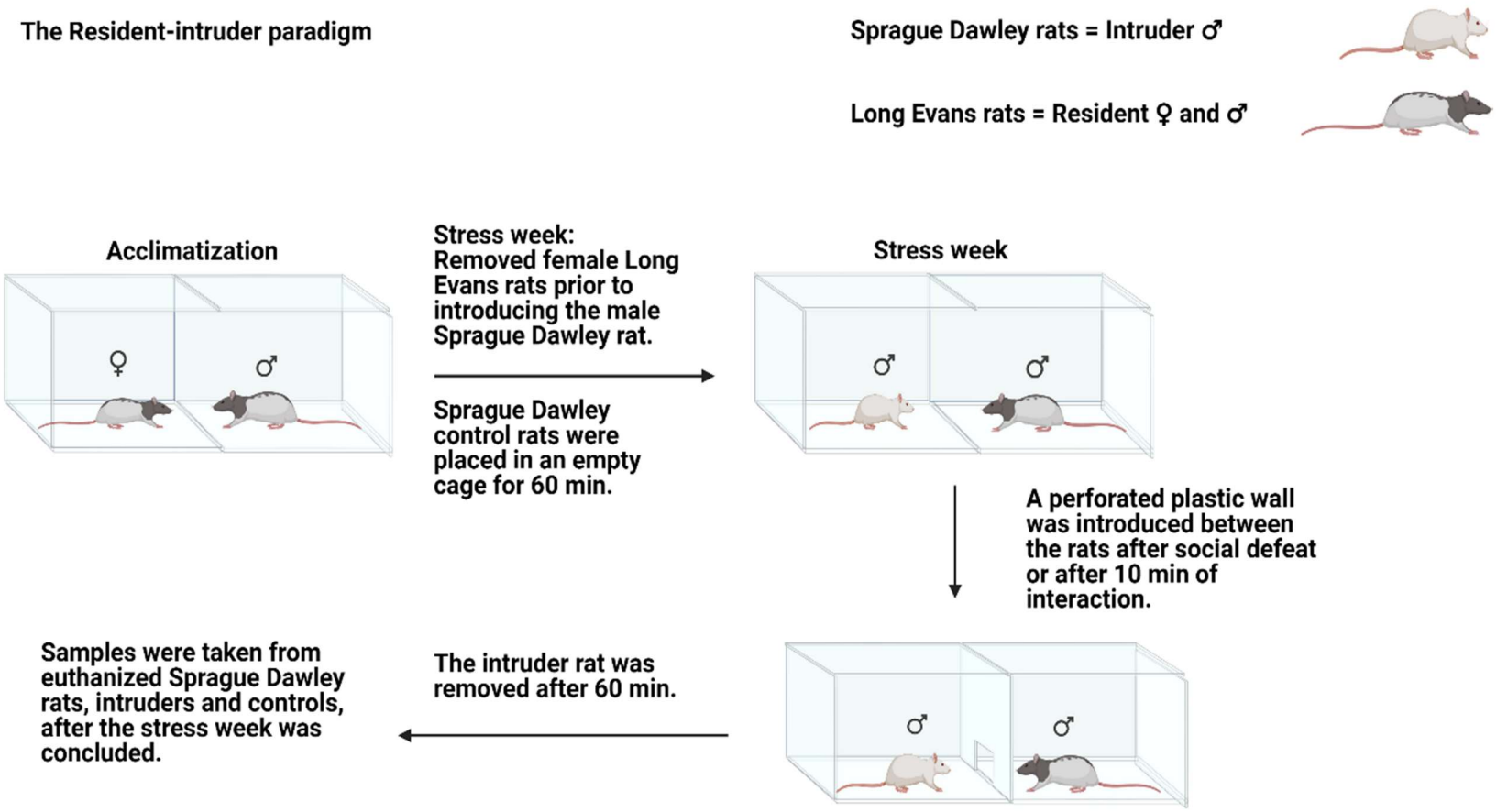


Figure 3. The resident-intruder paradigm [32]. This figure shows an overview of the resident-intruder paradigm with Sprague Dawley rats as intruders and Long Evans rats as dominant residents. This method is described in section 3.1. This figure was created with BioRender.com.

1.3.2 Genes investigated in the present thesis

Recent findings based on sequencing of RNA derived from the pituitary gland of five of the control Sprague Dawley rats and five of the stressed Sprague Dawley rats after the resident-intruder paradigm showed that some genes had a significantly altered gene expression [34]. Two of those genes were *Nrcam* and *Phf2,* that encode the proteins Neuronal Cell Adhesion Molecule and Plant Homeodomain Finger Protein 2, respectively. *Nrcam* was downregulated whereas *Phf2* was upregulated in the stressed rats.

For the present thesis, it was therefore decided to investigate if the two genes, *Nrcam* and *Phf2,* showed an altered gene expression in cells from a rat pituitary cell line, RC-4B/C, when exposed to CRH, mimicking stress. The RC-4B/C cells were also exposed to Dexamethasone (Dex), a synthetic CORT.

As described in section 1.2, CRH and CORT are both central hormones in the HPA axis. These two stress hormones were included in the present thesis to investigate whether CRH or Dex could lead to similar gene expression in the pituitary cells derived from a rat cell line as stress did in the stress exposed Sprague Dawley rats. Moreover, it was decided to assess the gene expression of *Skp1* in the CRH or Dex exposed RC-4B/C cells. The *Skp1* gene was included based on a possible co-expression of PHF2 and SKP1.

1.3.3 Genes possibly involved in the stress response

The *NRCAM* gene encodes the protein neuronal cell adhesion molecule (NRCAM), a protein in the L1 immunoglobulin superfamily that functions as a neuronal cell adhesion molecule [35]. This molecule has many diverse functions in the nervous system, particularly during development. NRCAM is involved in axon growth and guidance, the formation of synapses, and the formation of the myelinated nerve structure. NRCAM is interesting because this protein interacts with many molecules, both extracellularly and intracellularly. This could imply that a change in the structure or expression of NRCAM may affect many pathways since NRCAM can function as both a ligand and a receptor. Previous studies have shown an association between changes in the structure and/or expression of NRCAM protein and psychiatric disorders such as autism, alcohol dependency, and schizophrenia [35].

Recent data from our research group show that social stress may be associated with selected polymorphisms in the *NRCAM* gene and headache in humans [34].

Eukaryotic DNA exists in a packed state, called chromatin, that consists of nucleosome units packed together inside the nucleus (Figure 4). A single nucleosome consists of 147 base pairs of DNA wrapped around an octamer of core histones, where the core histones contain two molecules each of H2A, H2B, H3 and H4. The histone tails can be post-translationally modified to affect the regulation of gene expression, either repression or activation, by altering the accessibility of proteins to the packed DNA. Several covalent modifications on histone tails has been discovered, such as acetylation, methylation, phosphorylation, and ubiquitination [36, 37].The overall purpose of these modifications can be divided into two categories; 1) altering the state of chromatin, therefore altering the accessibility to DNA, and 2) creating a binding site for the recruitment of proteins [36, 37]. Proteins that have specific "reader" domains recognize the different modifications; e.g. Plant Homeodomain (PHD) finger protein 2 that uses its PHD domain to recognize methylated lysine [38, 39].

The *PHF2* gene encodes the Plant Homeodomain Finger Protein 2 (PHF2), which is a transcriptional regulator in the histone-lysine demethylase superfamily, that exerts its function by removing methyl groups on lysines located on histone tails [39]. The addition of methyl groups to lysine residues on histone tails is an important method of transcriptional regulation. The modification commonly occurs in histones H3 and H4 and the effects of the modification depend on which lysine residue the methyl group is attached to, and to which degree the methylation occurs [37].

PHF2 belongs to family of demethylases that have a catalytically active Jumonji- C domain (JmjC) depending on 2-oxoglutarate and Fe (II) to function as an enzyme. The PHD in PHF2 recognizes the target, methylated lysine, located on specific amino acid residues in histone tails [39]. The JmjC-domains on PHF2 can remove mono-and dimethyl groups on H3K9 (histone 3, lysine residue 9) and H3K27 (histone 3, lysine residue 27), and trimethyl-lysine on H4K20 (histone 4, lysine residue 20) [39]. Methylation on H3K9 is associated with repression of gene expression [37]. Consequently, PHF2 functions as an activating transcriptional regulator, due to the removal of the repressive methylation on H3K9 [39].

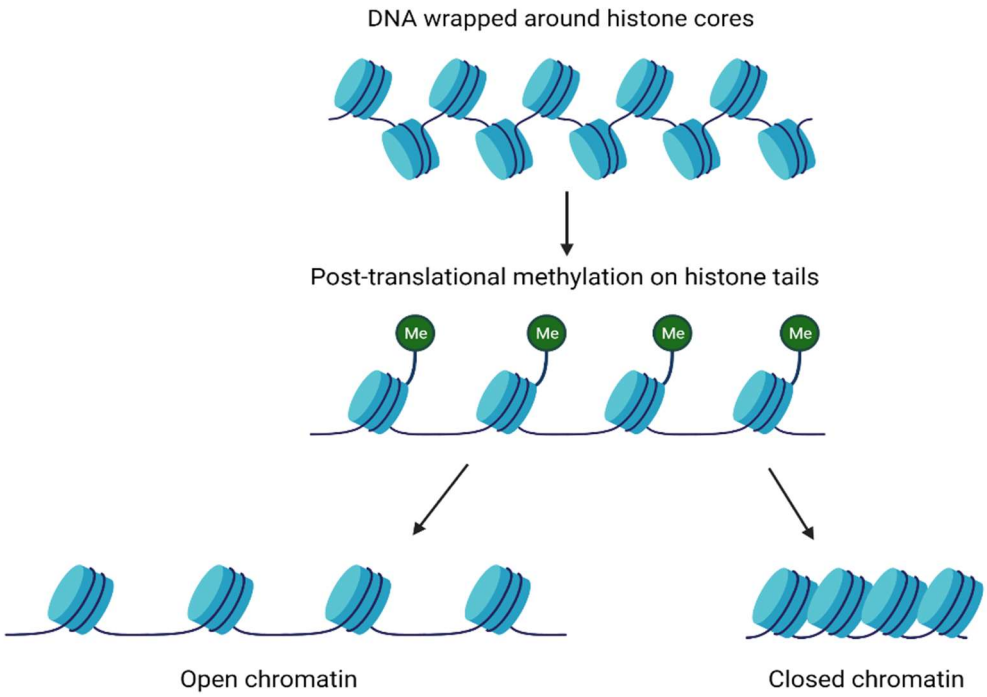


Figure 4. A schematic overview of the organization of nucleosomes into chromatin. Nucleosomes consist of ~ 150 bp of DNA wrapped around octamer histone cores that comprise of the histone core units H2A, H2B, H3 and H4, with two molecules of each unit. Post-translational methylation on histone tails can lead to either open chromatin or closed chromatin, consequently altering the accessibility of DNA. This figure was created with BioRender.com

PHF2 has been predicted to interact with SKP1 in *Homo sapiens* (Figure 5). The interaction score is 0.748, which is a description of how likely it is that an interaction is true, according to the STRING database [40]. The scores rank from zero to one, where one is the highest possible indicator of confidence [40]. The interaction between PHF2 and SKP1 is based on co-expression experiments [41, 40].

The STRING database also shows a predicted interaction between PHF2 and SKP1 in *Rattus norvegicus* [40] (Figure 6). However, this predicted interaction is based on the data from *Homo sapiens* (Figure 5). This means that there is no available experimental data to suggest a possible similar interaction between PHF2 and SKP1 in *Rattus norvegicus*.

The S-Phase Kinase Associated Protein 1 (SKP1), encoded by the gene *SKP1*, is a component of Skp1-Cullin1-F-box (SCF) ubiquitin E3 ligase complexes, where SKP1 functions as an adaptor protein, connecting protein-binding partners together. SKP1 recognizes and binds to proteins with F-box motif with the catalytic part of SCF complex. The F-box proteins bind to the substrates and thus functions as a receptor for the SCF complex [42]. The SCF complex is involved in ubiquination of specific substrates involved in a variety of different processes, such as cell cycle regulation, apoptosis, gene transcription, DNA replication, and tumorigenesis. A ubiquitination of the SCF substrates leads to a subsequent degradation of the substrate by a proteasome. Therefore, ubiquitination by the SCF complex is a highly important method for controlling the turnover of specific proteins in the cell [43, 42].

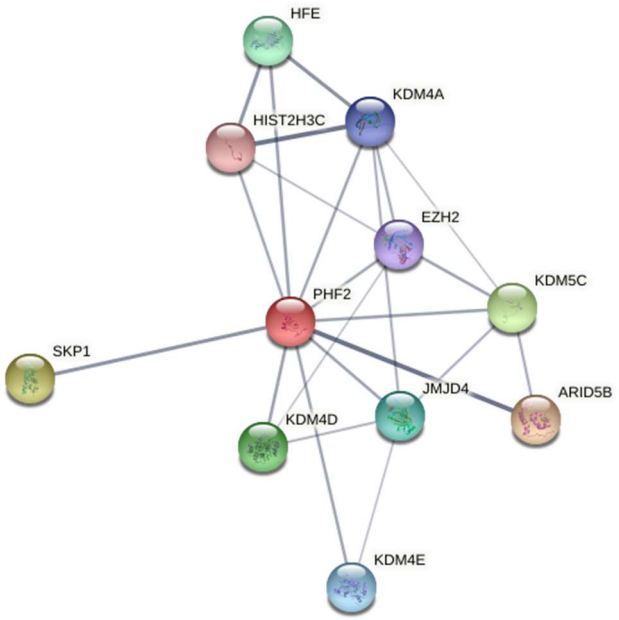


Figure 5.The interaction map of PHF2 in *Homo sapiens*. According to the STRING database, PHF2 (red node) has been shown via experimental data to be co-expressed with SKP1 (yellow node). This figure was retrieved from the STRING database [40].

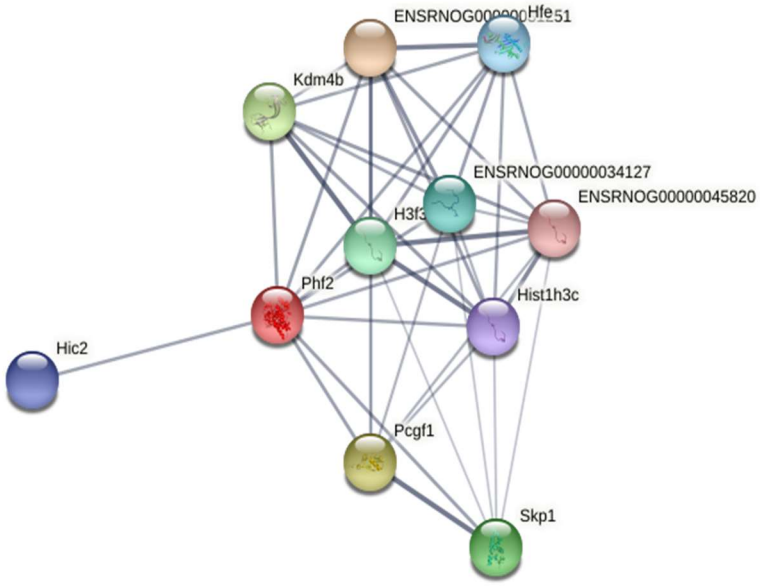


Figure 6. The interaction map of PHF2 in *Rattus norvegicus*. According to the STRING database, PHF2 is predicted to interact with SKP1. However, this data is based on the experimental data from *Homo sapiens* that show co-expression between PHF2 (red node) and SKP1 (green node). This figure was retrieved from the STRING database [40].

1.4 Human cohort

As previously described, stressors can exist in many forms, both physical and social, and may cause several adverse consequences. Social stress was investigated in the present thesis by assessing social stress defined as workplace bullying in a human cohort.

Our research team has previously conducted a study on humans where Statistics Norway (SSB), on behalf of the group, sent out a questionnaire to 5000 Norwegian employees between the ages of 18 to 60. Those who consented were sent a saliva kit. The subjects were requested to return the questionnaire along with a sample of their saliva to be used for genotyping [44]. The questionnaires and saliva kits were collected in three waves (T1–T3), and the data material used in the present thesis was from the first wave (T1). The first timepoint of data consists of questionnaires and saliva samples collected from 1226 subjects.

The questionnaire contained 37 questions regarding various aspects of the subject's work situation, such as the employer's leadership style and questions regarding bullying. The subjects were also asked to indicate their stress level, symptoms of anxiety and depression, and other negative behavioral problems in addition to physical problems such as headache, neck pain, and insomnia.

1.4.1 Workplace bullying

Workplace bullying is a serious concern among the working population, with an estimated prevalence between 10–15 percent in the European workforce [45]. Various researchers have defined workplace bullying over the years, one example is this definition by Matthiesen and Einarsen [46, 45]:

> *"Workplace bullying is a situation in which one or more persons systematically and over a long period of time perceive themselves to be on the receiving end of negative treatment on the part of one or more persons, in a situation in which the person(s) exposed to the treatment has difficulty in defending themselves against this treatment."*

The numerous consequences, both physical and psychological, of workplace bullying have been a subject for over 20 years of research [45, 47]. Examples of such consequences are; anxiety, depression, suicidal thoughts, headache, pain, and trouble sleeping [48, 47, 49, 33]. Due to the adverse effects that workplace bullying may cause, it is important to further investigate potential biological factors that may influence the stress response. This may in turn increase our understanding of the potential underlying biological mechanisms that can make subjects more vulnerable, or more resilient, to the adverse effects of bullying.

1.4.2 The CRHR1 gene

The *CRHR1* gene is located on chromosome 17q21.31[50]. The gene encodes the protein CRHR1, a GPCR that binds to its ligands; neuropeptides belonging to the CRH family. CRH, as described in section 1.2, is a peptide hormone secreted from the PVN in the hypothalamus, and its release initiates the HPA axis in response to stressful stimuli. Its receptor, CRHR1, is therefore a vital component in the stress response.

The receptor CRHR1 is highly expressed in certain areas in the brain, such as the cerebral cortex, cerebellum, amygdala, hippocampus, and the pituitary [51]. The consequence of such receptor-ligand binding depends on the tissue. CRHR1 has been shown to bind different G proteins in different tissues, thereby activating tissue-specific signaling pathways [51]. The emphasis in the present thesis is the stress response and the stress-related signaling pathway of the corticotrophs cells in pituitary tissue. When the agonist, the peptide hormone CRH, binds to CRHR1, a conformational change occurs in the receptor allowing it to activate trimeric G proteins and consequently transduce downstream signals [51].

The downstream signaling leads to an activation of the cyclic adenosine monophosphate (cAMP)/Protein kinase A (PKA) pathway. This activation has two consequences. The first is the release of ACTH from vesicles near the cell membrane, which leads to an exocytosis of ACTH into the circulation. This occurs due to rapid phosphorylation of ion channels and increased calcium influx [52, 19]. The second consequence is the transcription of the *POMC* gene. Due to the immediate release of ACTH, the storage of ACTH containing vesicles must be replenished. This is achieved in a couple of different ways downstream from the activated cAMP/PKA signaling pathways. One of the ways is by phosphorylation of the cAMP response element-binding protein (CREB), a transcription factor that can initiate transcription of *POMC* [19]. The Mitogen-activated protein kinase (MAPK) signaling pathway and the nuclear orphan receptor Nu77 is another way to activate transcription of the *POMC* gene [53, 19]. Regardless of the pathway, the end result is transcription of *POMC* and a subsequent synthesis of ACTH.

Dysregulated levels of the ligand for CRHR1, CRH, has been linked to anxiety, depression, trouble sleeping, chronic pain, and addictive behaviors such as alcohol dependency [51]. Since CRHR1 mediates the stress effects of CRH, it is tempting to speculate that it could also involve primary receptors in the brain.

1.4.3 Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) is a phenomenon that occurs approximately one in 1,000 base pairs where one nucleotide has different variants; this is the most common source of genetic variation in a population. A SNP may lead to an amino acid change if the SNP is in a codon (nonsynonymous), but it can also be silent (synonymous) or occur in the noncoding regions of the gene. Moreover, SNPs may affect gene expression, messenger RNA (mRNA), and proteins through various means, e.g. promoter activity [54]. Combinations of SNPs that tend to be inherited together are in a linkage disequilibrium (LD), which means there is a nonrandom association of the SNPs [55]. A set of SNPs that are in high LD is known as a haplotype, and they are inherited as a unit. As humans are diploid organisms, each person has two haplotypes. Furthermore, each SNP has a major and a minor allele, and each haplotype is a combination of major and minor alleles [56].

In the present thesis, the haplotype of interest is in the *CRHR1* gene, which consists of a combination of the three SNPs; rs7209436, rs110402 and rs242924. The three SNPs are found within the intronic region in the *CRHR1* gene and they are in high LD [57-59]. Previous studies have shown an association between intronic regions and regulation of gene expression [60], it therefore is not unlikely that the three SNPs might be involved in the regulation of *CRHR1* gene [61]. Moreover, previous studies have shown an association between the three SNPs and an altered expression of CORT [57, 62-64].

1.4.4 CRHR1 haplotype

The combination of the SNPs rs7209436, rs110402 and rs242924 form a haplotype, where CGG and TAT are the most frequent haplotype alleles. The TAT haplotype has been the subject of many research studies [61, 65, 62, 66, 58, 67]. Some of the studies have discussed the potential protective effect of the TAT haplotype against depression in individuals who have experienced early life stress in the form of childhood maltreatment [61, 65, 62, 58].Thus, one of the aims of the present thesis was to examine: does the TAT haplotype have a protective effect against symptoms of depression if the subject has been exposed to workplace bullying?

In a study by Bradley et al. [61], the authors sought to investigate whether the effects of childhood maltreatment on depression in adulthood was moderated by SNPs within the *CRHR1* gene. The subjects were mainly African Americans, with a smaller control group that consisted of Caucasian women. Childhood maltreatment for the subjects was assessed by using a Childhood Trauma Questionnaire (CTQ). The authors discovered that rs110402, rs7209436 and rs242924, who form the TAT haplotype, had the strongest gene x environment interaction effects. The TAT haplotype was overrepresented in subjects that did not show major depression disorder (MDD) when exposed to moderate or severe childhood maltreatment. The authors state that their results support the hypothesis regarding the CRHR1 system's involvement in moderating the effect of childhood maltreatment on depression in adults, by mechanisms such as polymorphisms that may alter the activity of the CRHR1 system.

In a study by Polanczyk et al. [65], the authors further examined the hypothesis proposed by Bradley et al. [61], which theorized a theoretical protective effect of the TAT haplotype against adult depression when the subjects were previously exposed to childhood trauma. The hypothesis was tested using two parallel studies that were conducted in two different countries, one in England and one in New Zealand. The results were ambiguous; in the England study, the TAT haplotype was associated with a protective effect against MDD. However, the result was not replicated in the New Zealand study. The authors stated that the main difference between these two studies was that the England study used the CTQ to assess childhood maltreatment whereas the New Zealand study used a different method to assess childhood maltreatment.

In a study by Tyrka et al. [62], the authors formed a hypothesis based on the studies by Bradley et al. and Polanczyk et al. The hypothesis was whether polymorphisms in *CRHR1* would interact with childhood maltreatment to predict HPA axis reactivity. The authors tested two of the three SNPs investigated in the present thesis, rs110402 and rs242924, and found that both of these SNPs had a significant interaction with childhood maltreatment. The authors tested the cortisol response in the subjects by using a Dex/CRH test over two days. During the test, Dex, a synthetic cortisol, was given the day before the subjects had an intravenous (IV) inserted. The subjects were then given CRH and had their blood drawn repeatedly over the next hours. Cortisol levels were measured in the blood samples. For subjects with no history of moderate to severe maltreatment, there was no variation in the cortisol response regardless of the genotype. For the subjects that had reported a history of moderate to severe maltreatment the result was different; the GG genotype of both SNPs was associated with a higher cortisol response after the Dex/CRH test. A higher cortisol response has been linked to a dysregulated HPA axis, and in particular, symptoms of depression [68].

However, some studies disagree with the hypothesized protective effect of the TAT haplotype. In a study by Davis et al. [67], the effect of the TAT haplotype was assessed in a cohort that consisted of subjects with MDD. The authors found that the subjects with more copies of the TAT haplotype had more cognitive issues associated with depression, compared to subjects with zero copies of TAT haplotype. Two symptoms of depression were specifically associated with the TAT haplotype: indecisiveness and rumination.

A study by Kranzler et al. [58] partially supports the protective effect of the TAT haplotype. However, the study also partially supports the evidence against TAT as a protective haplotype. The authors found that subjects with the TAT haplotype had a significantly lower risk of experiencing a depressive episode when exposed to adverse events in their childhood. However, these results were exclusively found in African American women. Furthermore, they also found that in African American women who had no childhood trauma but had two copies of the TAT haplotype, the risk of a major depressive episode was approximately 50 percent greater. They also included African American men and European American women and men in the study, but the results were not significant for any of these sample populations.

Moreover, a study by Grabe et al. [66] identified an association between the TAT haplotype and childhood physical neglect. However, the TAT haplotype did not have a protective effect against depression in adults, but quite the opposite, the subjects with the TAT haplotype had increased symptoms of depression.

Due to these contradicting studies, it seems like there is no clear consensus regarding the hypothesized effect of the TAT haplotype.

In the present thesis we sought out to investigate whether the haplotype characterized by three selected SNPs in the *CRHR1* gene (rs7209436, rs110402, rs242924) could moderate the outcome, symptoms of depression, when the subjects were exposed to social stress in the form of workplace bullying. The *CRHR1* gene was selected due to the receptor's significance in the initiation of the HPA axis response.

2. Aims

Previous studies show that stressors may affect gene expression in the brain stem. In particular, gene expression relating to pathways downstream from the pituitary gland, in both animals and humans [69]. Hence, exposure to stress may induce the fight or flight response, but also social withdrawal and depression. The purpose of the present thesis was, 1) to investigate stress reactions in a cell line from a rat pituitary, and 2) to study the association between social stress, genetic susceptibility, and symptoms of depression in a human cohort.

More specifically, the thesis aimed to:

Ia. Explore the release of ACTH in the cell medium; the ACTH was measured in the cell medium from the RC-4B/C cells (rat pituitary gland) following exposure treatment with the stress hormone CRH.

According to the function of the HPA axis, exposing the cells to CRH should trigger the release of ACTH into the cell medium.

Ib. Investigate the gene expression in the RC-4B/C cell line, i.e. examine the expression of *Nrcam*, *Phf2* and *Skp1* induced by the stress hormones CRH and synthetic CORT, Dex.

The RC-4B/C cell line isolated from rat pituitary gland should be ideal to stimulate with CRH and Dex because the pituitary gland is a central component in the HPA axis.

II. Address the role of *CRHR1* gene haplotype (rs7209436 T/C, rs110402 A/G and rs242924 T/G) TAT versus CGG regarding stress-induced symptoms of depression.

The investigation required a stepwise approach, first single SNP genotyping, followed by haplotyping using the Phase software. The outcome, symptoms of depression, was measured with a questionnaire based on the Hopkins checklist.

3. Materials and Methods

3.1 *In vitro* arm*;* The rat model and the RC-4B/C cell line

3.1.1 The Resident-intruder paradigm

This is a description of the Resident-intruder paradigm method used by the research team at STAMI to expose Sprague Dawley rats to chronic social stress in order to mimic chronic social stress in humans [32].

The purpose of the resident-intruder paradigm is to study the stress response in rats by exposing intruder rats to dominant resident rats. The rat strain Long Evans were used as the dominant resident and the Sprague Dawley strain were used as the intruder rat. A total of ten male and ten female Long Evans rats were included in the study, these were placed together in pairs, one male and one female per cage: weighing 500–550 g and 250 g, respectively. Sprague Dawley rats (100–400 g), which served as intruder rats, were divided into two groups with ten rats in each group. The respective groups were the exposed/stress group and the control group, each group were housed together in pairs. The two different rat strains were kept in different rooms.

Each day during the stress week, the female Long Evans rat was removed one hour before the stress exposure, and a male non-dominant Sprague Dawley intruder rat was placed in the cage along with the dominant male Long Evans resident rat. A perforated plastic wall was introduced between the two rats after a social defeat or after ten minutes of interaction. The perforated plastic wall allowed the rats to sense each other through vision, smell, and hearing; as a result, the stressor was still present. The intruder rat was removed after 60 minutes and returned to its own cage. The control rats were not introduced to a resident rat but were instead relocated to an empty cage for 60 minutes. The stress week lasted 7 days, and the intruder rats were introduced to a new dominant resident animal each day.

All the rats, both controls and intruders, were euthanized after the stress week. Samples were only collected from the Sprague Dawley rats. Blood samples were collected from the heart, and the pituitary gland and adrenal glands were harvested.

3.1.2 RC-4B/C Cell culture

The RC-4B/C (ATCC® CRL1903™) pituitary adenoma cell line isolated from rat, *Rattus norvegicus,* was used in the cell experiments [70, 71]. A pituitary adenoma is a benign tumor [72]. The RC-4B/C cell line contains cells that produce Luteinizing hormone (LH), Growth hormone (GH), Follicle-stimulating hormone (FSH), prolactin, ACTH, and thyrotropin beta, where ACTH is of interest in the present thesis.

The cells were grown in a medium specified for the cells, the components are listed in table 11 in Appendix A. The protocol for medium change and subculturing the RC-4B/C cell line is listed in Appendix A.

3.1.3 The Lactate Dehydrogenase cytotoxicity assay

The Lactate Dehydrogenase (LDH) cytotoxicity assay is a method used to measure if a certain treatment harmed the cells, causing cell membrane to leak. The principle of the method is that the plasma membrane of the damaged cells will be leaky. This leaky plasma membrane will lead to a release of the enzyme LDH which is normally present in the cytosol of most cell types. Its function is to interconvert lactate into pyruvate and, at the same time, reduce NAD^+ into NADH [73]. Any leakage of LDH into the cell medium can be measured after adding the enzyme Diaphorase. This enzyme uses the reduced NADH to reduce tetrazolium salt (INT) into a Formazan product, which can be detected at 490 nm (Figure 7). The Invitrogen™ CyQUANT™ LDH Cytotoxicity Assay Kit was used in the present thesis, and the absorbance was measured using a SpectraMax spectrophotometer.

The LDH cytotoxicity assay was used to assess whether the concentrations of CRH (1000 nM, 4000 nM) and Dex (1000 nM) that was used during the cell culture exposure experiments (section 3.1.4) were toxic to the RC-4B/C cells.

The detailed protocol is listed in Appendix B.

The result of the LDH cytotoxicity assay is given in percentage cytotoxicity and can be found in the Supplementary section as Supplementary figure 1.

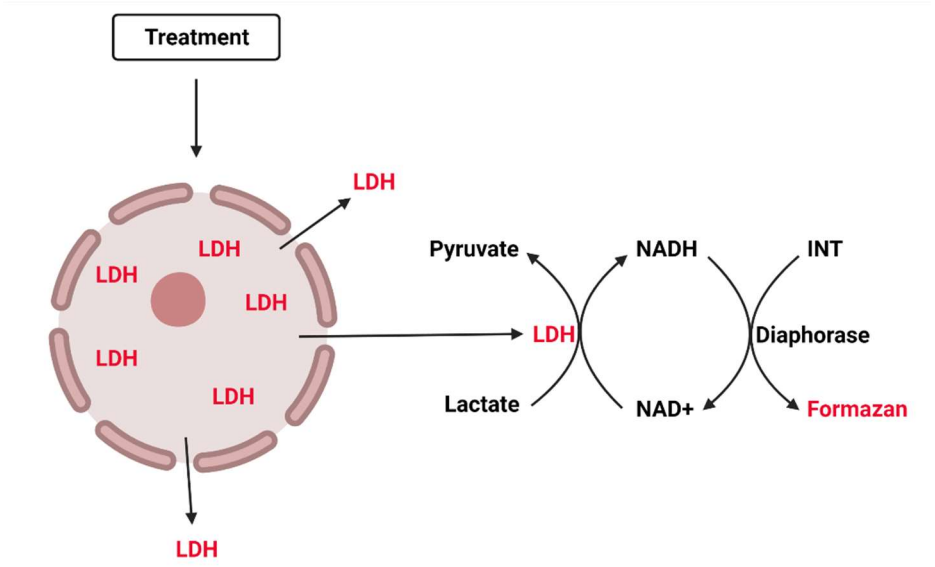


Figure 7. Overview of the principle behind the LDH cytotoxicity assay. Damaged cells leak LDH, an enzyme that can reduce NAD+ into NADH. The reduced NADH is used by the enzyme Diaphorase to convert INT into a red compound, Formazan. Formazan can be detected at 490 nm using a spectrophotometer. This figure was created with BioRender.com.

3.1.4 Cell culture exposure experiments

The RC-4B/C pituitary cells were exposed to CRH or Dex. The experiments were conducted according to the protocol in Appendix C.

The goal of the exposure experiments was to simulate the effects of the HPA axis in the cells. As described in section 1.2, CRH is released from the hypothalamus and leads to a subsequent release of ACTH from the pituitary gland. After exposure to CRH, the medium of the RC-4B/C pituitary cells was therefore analyzed for the presence of ACTH by using an immunoassay. RNA was isolated from cells collected 1 hour and 3 hours after exposure to CRH or Dex and was later used to investigate the expression of selected genes by RT-qPCR.

Cells were exposed to either one of two different concentrations of CRH, 1000 nM and 4000 nM, or to 1000 nM Dex. Cells were incubated with CRH for 20 minutes, 1 hour and 3 hours or with Dex for 1 hour and 3 hours. Control cells were incubated with medium only for corresponding incubation times. Cell media were collected after 20 minutes, 1 hour and 3 hours whereas cells were harvested after the 1 hour and 3 hours only. The experiments were conducted between passage 12–21. See overview in figure 8.

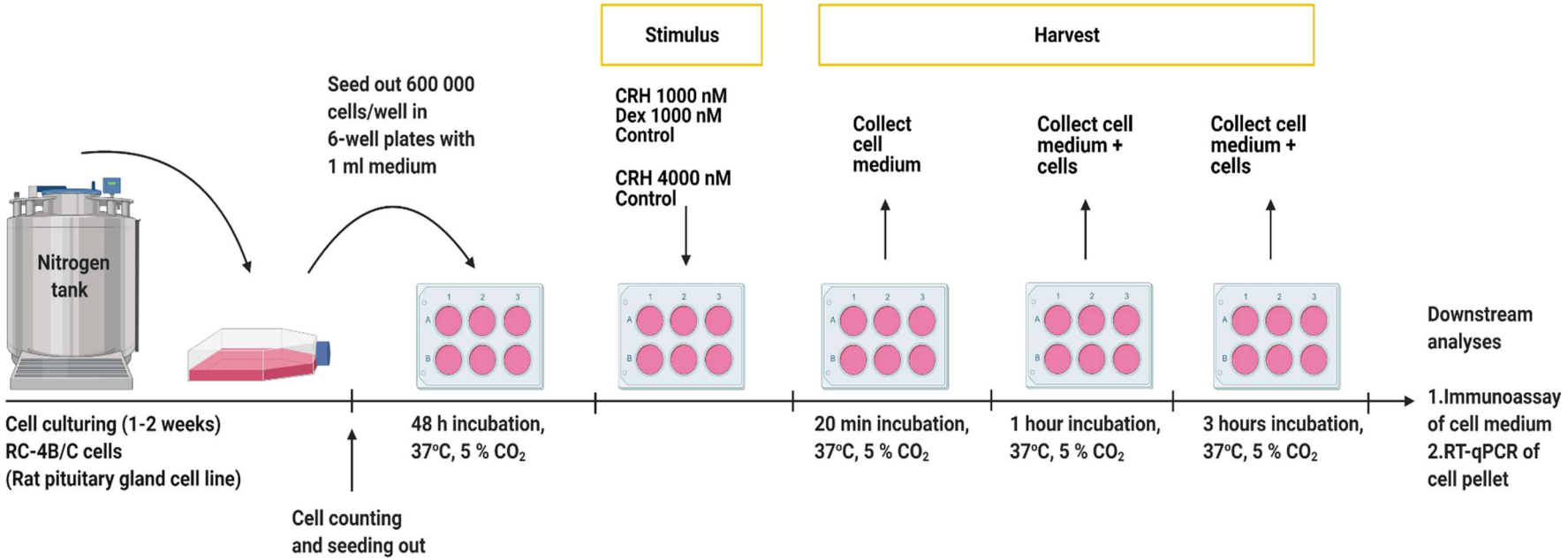


Figure 8. The cell culture exposure experiments – an overview. RC-4B/C cells were taken out of the nitrogen tank freezer and passaged in cell culture in about 1–2 weeks before the experiments started. The RC-4B/C cells were exposed to CRH (1000 nM, 4000 nM) or Dex (1000 nM). The experiments were conducted over a period of 5 weeks. This figure was created in BioRender.com

3.1.5 RNA isolation

RNA was isolated from the RC-4B/C cells using the RNA/DNA Purification Kit from Norgen Biotek (Cat. No. 48700). The RNA was isolated from cells exposed to CRH or Dex, as described in section 3.1.4. The cells were lysed with an SKP buffer from the RNA/DNA purification kit prior to the isolation, as the final step of the cell culture exposure experiment. A detailed protocol is listed in Appendix D.

The isolated RNA was further used in the present thesis as template for synthesis of complementary DNA.

3.1.6 Measuring RNA concentration

The concentration of the isolated RNA was measured using a Thermo Fisher Scientific NanoDrop 2000 spectrophotometer. Elution solution A from the Norgen Biotek RNA/DNA purification kit was used to adjust for background.

3.1.7 Synthesis of complementary DNA

The synthesis of complementary DNA (cDNA) was achieved by reverse transcription of the RNA template, using the qScript® cDNA Synthesis Kit from Quantabio®. The RNA isolated from RC-4B/C cells in section 3.1.5 was used as a template for the synthesis of complementary DNA. The cDNA reaction was performed on a Mastercycler from Eppendorf®.

A detailed protocol is listed in Appendix E.

3.1.8 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) is a method for measuring quantitative gene expression levels. Primer sequences for selected genes are used as probes to amplify desired gene regions. The template for the RT-qPCR was the cDNA synthesized in section 3.1.7. The RT-qPCR method was used to examine the gene expression of the following genes in the RC-4B/C cells following exposure to CRH or Dex: *Nrcam, Phf2* and *Skp1*. The housekeeping genes Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and *β-actin* were both tested as potential endogenous controls, however, only *Gapdh* were further used as endogenous control on the samples.

The RT-qPCR was performed on a QuantStudio5 machine from Thermo Fisher Scientific. The primers for the genes *Nrcam, Phf2* and *Skp1* were pre-designed primers from Sigma-Aldrich, and the primer for the *Gapdh* gene was a pre-designed primer from Bio-Rad. The primer sequences for the primers from Sigma-Aldrich are listed in table 1, along with their length and melting temperature. The primer sequence for *Gapdh* was not available, only the amplicon context sequence, listed in table 2. Standard curves were obtained for each primer to determine the optimal input of cDNA for each primer, and to check that all the primers used were equally efficient. All samples were run in triplicates.

Analysis of the RT-qPCR results was performed using the QuantStudio™ Design & Analysis Software (Thermo Fisher Scientific) and the data were further was processed in Microsoft Excel. The ΔΔCt (delta-delta Ct) method was implemented on the results to determine the gene expression level. The data were normalized to the expression of the reference gene, *Gapdh*.

For the statistical analyses, one-way ANOVA was implemented on the data from the samples that had been exposed to 1000 nM CRH or 1000 nM Dex. The Mann-Whitney rank sum test was implemented on the data from the samples exposed to 4000 nM CRH. The results were considered statistically significant if the p-value was < 0.05. The results are listed in Supplementary figure 1 and 2.

The protocol for the RT-qPCR method is listed in Appendix F.

Table 1. The selected primers for the qPCR of the RC-4B/C cells and their sequence, length and melting temperature.

PRIMERS	SEQUENCE 5'→ 3'	BASE PAIRS	TM ºC
NRCAM FORWARD	GGATAACTCCTTTCAAAGACTG	22	58.4
NRCAM REVERSE	ATAGCAGATGTAGTCCTCAC	20	53.1
PHF2 FORWARD	AAGCATTCAAAGGTTCTCAC	20	58.4
PHF2 REVERSE	ATGACCTAAAAGCACCATTG	20	59.1
SKP1 FORWARD	CTTTACTGAAGAGGAGGAGG	20	57.1
SKP1 REVERSE	TCTGATCTGTACTGGAACTAC	21	53.2

Table 2. The GAPDH primer: amplicon context sequence, length and melting temperature.

PRIMER	AMPLICON CONTEXT SEQUENCE	BASE PAIRS	TM ºC
GAPDH	TGATGGCAACAATGTCCACTTTGTCACAAGAGAA GGCAGCCCTGGTAACCAGGCGTCCGATACGGCC AAATCCGTTCACACCGACCTTCACCATCTTGTCT ATGAGACGAGGCTGGCACTGCACAAGAAGATGC GGCTGTCTCTA	115	85.5

3.1.9 ACTH immunoassay

ACTH levels in cell media isolated from RC-4B/C cells exposed to CRH (1000 nM/4000 nM), section 3.1.4, were assessed using an immunoassay.

The Luminex xMAP® technology uses magnetic beads that are colored with a unique mix of fluorophores for identification by lasers. Specific antibodies for each protein of interest are coupled to the beads and several beads can be used simultaneously to allow detection of several proteins from one sample. Detection antibodies with conjugated Biotin are added along with Streptavidin PE substrate. Addition of the detection antibodies with Streptavidin PE makes it possible to detect and quantify the proteins [74]. An overview of the method can be seen in figure 9.

The kit used for detection of ACTH was Milliplex® MAP Rat Stress Hormone Magnetic Bead Panel from Merck®. The assay was performed on Bio-Plex® MAGPIX™ Multiplex Reader, with the software Bio-Plex Manager MP.

The protocol for the ACTH immunoassay method is listed in Appendix G.

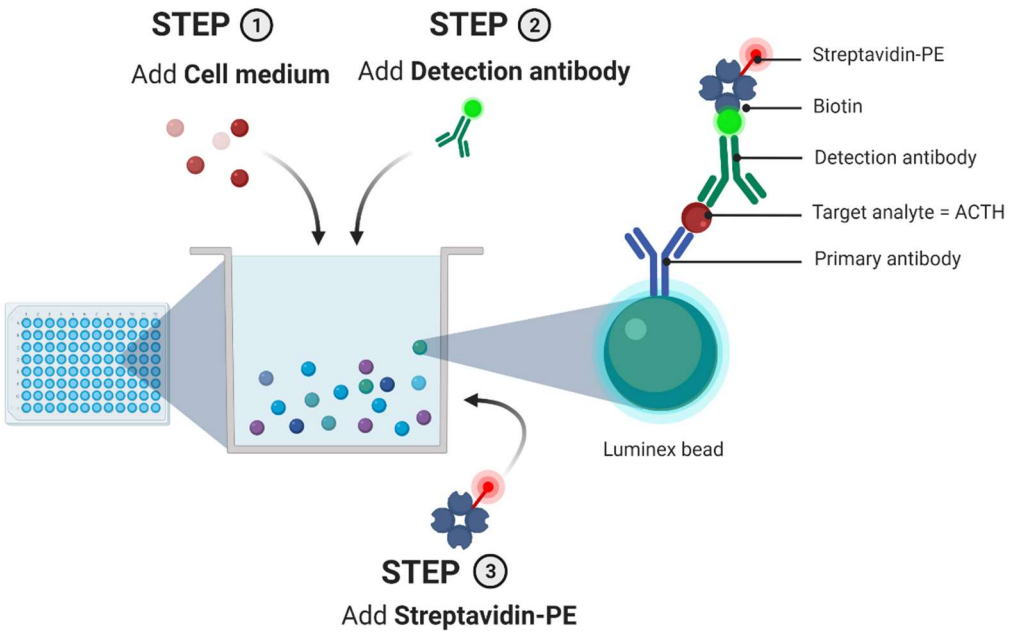


Figure 9. Overview of the principle behind the Luminex immunoassay. The isolated cell medium that contains the analyte is mixed with primary antibodies that targets the analyte, ACTH. Detection antibodies along with Streptavidin-PE is added to detect and quantify the analyte. This figure was created in BioRender.com

3.2 The Human arm; general population

3.2.1 Genotyping

Genomic DNA was previously isolated from saliva samples provided by subjects in a randomized selection of the working population in Norway. Samples were collected at three different timepoints, the presented data were analyzed by genotyping from the first collected timepoint (T1). From this human cohort of 1226 samples, genomic DNA was isolated and genotyped, where 1184 samples were used in the downstream analyses. Single nucleotide polymorphism genotyping was conducted using TaqMan SNP genotyping assays (Thermo Fisher Scientific).

Three SNPs for the gene Corticotropin Releasing Hormone Receptor 1 (*CRHR1*) were analyzed; rs7209436, rs110402 and rs242924. The TaqMan genotyping master mix and assay mix from Thermo Fisher Scientific contained the respective probes and primers that was used in the protocol. The reporter dyes FAM and VIC were used to label the probes to distinguish between the two allele variants for each SNP, as listed in table 3. The genotyping was performed on a QuantStudio 5 machine with software from Thermo Fisher Scientific. The information regarding which allele variants to detect in the software was retrieved from Thermo Fisher Scientific [75].

The protocol for genotyping is listed in Appendix H.

Table 3. The CRHR1 SNPs and their allele variants.

CRHR1 SNP	ALLELE	ALLELE
RS7209436	C	T
RS110402	G	A
RS242924	G	T

3.2.2 Haplotyping

The software PHASE v2.1.1 [76] was used to reconstruct the different haplotypes from the three SNPs; rs7209436, rs110402 and rs242924. The haplotyping of the *CRHR1* SNPs was based on the results from the genotyping of 1184 saliva samples.

3.2.3 Statistics

The data utilized in the present thesis consisted of questionnaires collected previously [44], and new data from the genotyping and subsequent haplotyping. The statistics program IBM SPSS 25 was used for organization of the data, whereas the statistical analyses were conducted using Stata SE 16.0. The results were considered statistically significant if the p-value was < 0.05.

Linear regression analyses were used to assess any association between independent and dependent variables. The variables in the present thesis were bullying in the workplace, mean symptoms of depression, based on the Hopkins symptom checklist (HSCL) [77], and the CRHR1 haplotype (Figure 10).

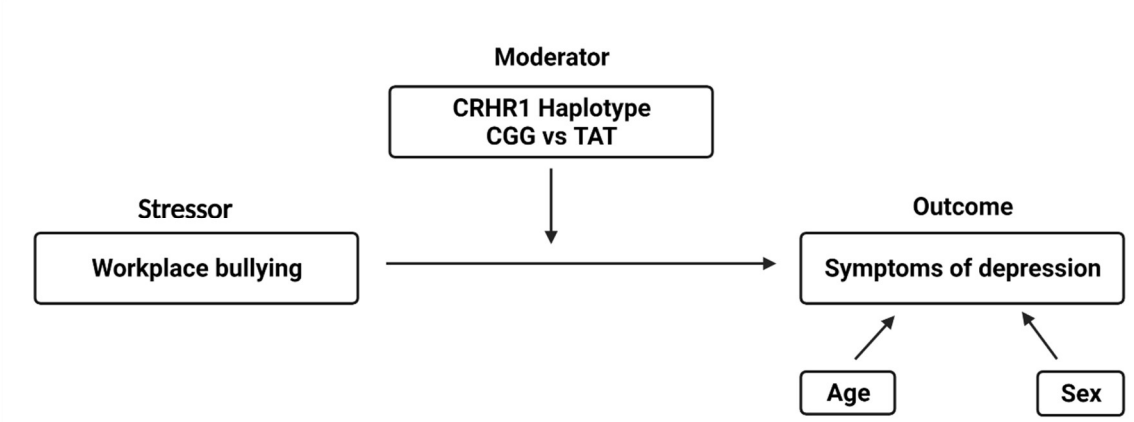


Figure 10. Schematic illustration of the proposed model. Illustrating the proposed relationship between the stress exposure (workplace bullying), moderator (CRHR1 haplotype), and the outcome (symptoms of depression).

3.2.3.1 The statistical variables

The subjects were asked to state whether they had experienced bullying in the workplace (Figure 11). The alternatives were “No”, “Yes, rarely”, “Yes, sometimes”, “Yes, several times a week” and “Yes, daily”. The answers were dichotomized into two groups with the cut off between “No” and “Yes, rarely”. This was done to create two clearly defined groups; those exposed (to any degree) and those who were not.

Bullying as a term was defined in the questionnaire just above the question. It was defined as (translated from Norwegian):

“Bullying (for example, harassment, torment, ostracism, or hurtful teasing and joking) occurs when a person is repeatedly subjected to unpleasant, degrading, or hurtful treatment in the workplace. In order for us to be able to call something bullying, it must take place over a certain period of time, and the person who is being bullied must have difficulty defending himself. It is not bullying if two equally "strong" people come into conflict or it is only a single episode.”

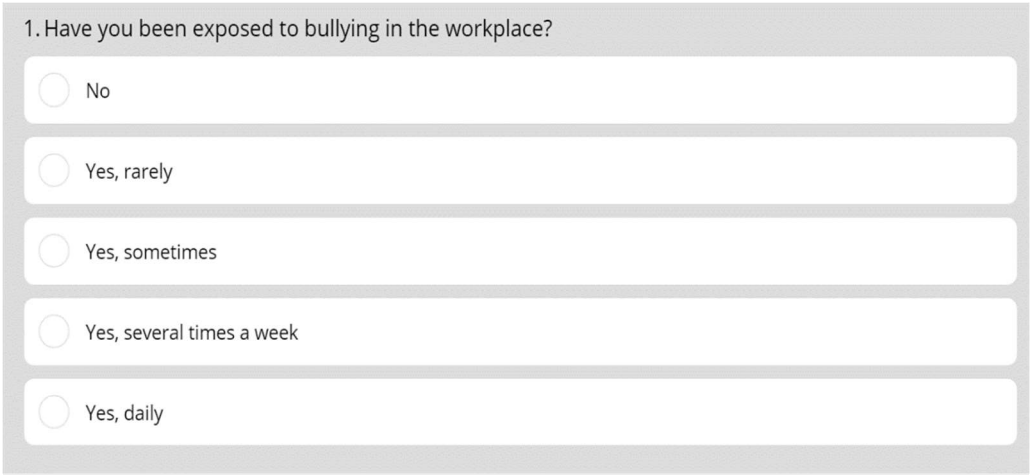
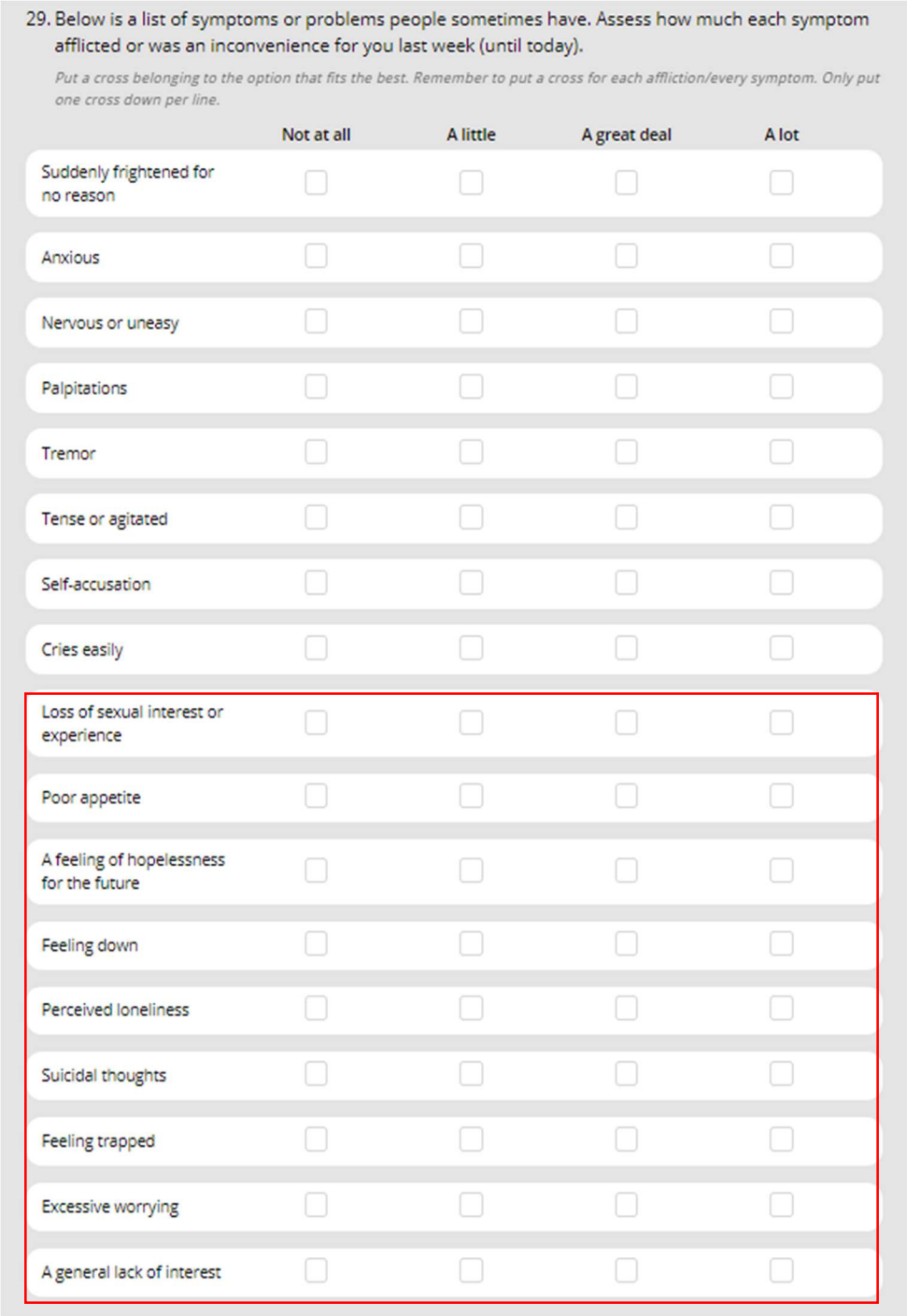


Figure 11. Bullying in the workplace. This figure shows a translated version of the question regarding exposure to bullying in the workplace from the questionnaire given to the subjects (section 1.4).

The outcome was measured in various symptoms of depression. Figure 12 shows the translated version of question 29 from the questionnaire, regarding the subjects' experience related to symptoms of anxiety and depression. These questions are based on a modified short version of the HSCL [78]. There are seventeen questions regarding various aspects of the subject's mental state. However, the included items that were used to measure symptoms of depression in the present thesis was the bottom 9 questions, see red box figure 12. This cutoff was based on the distinction between symptoms of anxiety and depression based on the HSCL [77]. There were four response categories; "Not at all", "A little", A great deal" and "A lot" and the mean depression variable was created by calculating the average values from 1–4. A mean variable was therefore created from these questions representing symptoms of depression in Stata SE 16.



29. Below is a list of symptoms or problems people sometimes have. Assess how much each symptom afflicted or was an inconvenience for you last week (until today).

Put a cross belonging to the option that fits the best. Remember to put a cross for each affliction/every symptom. Only put one cross down per line.

	Not at all	A little	A great deal	A lot
Suddenly frightened for no reason	☐	☐	☐	☐
Anxious	☐	☐	☐	☐
Nervous or uneasy	☐	☐	☐	☐
Palpitations	☐	☐	☐	☐
Tremor	☐	☐	☐	☐
Tense or agitated	☐	☐	☐	☐
Self-accusation	☐	☐	☐	☐
Cries easily	☐	☐	☐	☐
Loss of sexual interest or experience	☐	☐	☐	☐
Poor appetite	☐	☐	☐	☐
A feeling of hopelessness for the future	☐	☐	☐	☐
Feeling down	☐	☐	☐	☐
Perceived loneliness	☐	☐	☐	☐
Suicidal thoughts	☐	☐	☐	☐
Feeling trapped	☐	☐	☐	☐
Excessive worrying	☐	☐	☐	☐
A general lack of interest	☐	☐	☐	☐

Figure 12. 17-item version of the Hopkins symptom checklist. This figure shows a translated version of the questions that pertains to the subjects' experience of anxiety and depression. The focus area in the present thesis is the symptoms of depression, which only includes the last 9 questions in this figure (red box).

3.2.4 Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium (HWE) can be defined as [79]:

> *"Hardy-Weinberg equilibrium (HWE) is the state of the genotypic frequency of two alleles of one gene locus after one generation of random mating in an indefinitely large population with discrete generations, in the absence of mutation and selection. "*

The human population is usually genetically stable which means that the frequencies of the alleles are unaltered; described as a genetic equilibrium. It is, therefore, important to test HWE on each SNP tested to assess for any deviations from the equilibrium. If disequilibrium is seen it could be a sign of an error relating to lab work (genotyping) or selection of the cohort. Other reasons can be i.e. natural and artificial selection, migration, emigration, mutations, inbreeding, or assortative mating [79].

Deviation from HWE is measured by using a Chi square score and a p-value. The Chi square is a method that compares the observed genotype counts with the expected genotype counts. When testing HWE on a sample population the p-value should not be significant.

4. Results

4.1 The RC-4B/C cell line

4.1.1 Luminex

The results of the immunoassay on the RC-4B/C cell media showed no clear effects of the CRH stimulation of cells on the ACTH media concentration. Low levels of ACTH were detected, and unfortunately, the cell media had an ACTH concentration close to the background medium control. Figure 13 A shows the standard curve. The concentrations of ACTH in both CRH-exposed samples and controls ranged between 2–10 pg/mL.Moreover, the concentration for several samples, both exposed and controls, were out of range. The concentration for the controls were in the same concentration range as the exposed samples, as seen in figure 13 B and C. The group mean of the concentration for samples exposed to 1000 nM CRH or 4000 nM CRH can be seen in figure 13 B and C, respectively. The cell media isolated from control cells and the cells exposed to 1000 nM CRH (Figure 13 B) showed a higher concentration of ACTH after three hours compared to the concentration after 20 minutes and one hour. The ACTH concentration in the cell medium of cells exposed to 4000 nM CRH and controls was approximately the same for the three time points (Figure 13 C).

4.1.2 RT-qPCR

The results of the RT-qPCR after stimulation of RC-4B/C cells with 1000 nM CRH or 1000 nM Dex showed no significant effects of CRH and Dex exposure, respectively, on the gene expression of selected genes compared to controls (Figure 14). The accompanying p-values for samples exposed to 1000 nM CRH or 1000 nM Dex are listed in supplementary table 1. Only a weak trend towards a possible downregulation after three hours of exposure to CRH or Dex was observed for the three target genes: *Nrcam*, *Phf2* and *Skp1* (Figure 14 B, D and F). No such trend of downregulation can be seen after one hour of CRH or Dex stimulation (Figure 14 A, C and E). Exposure to 4000 nM CRH showed no significant effects on the gene expression of *Nrcam*, *Phf2* and *Skp1* in the cells exposed to 4000 nM CRH compared to controls; see supplementary figure 2 and accompanying p-values listed in supplementary table 2.

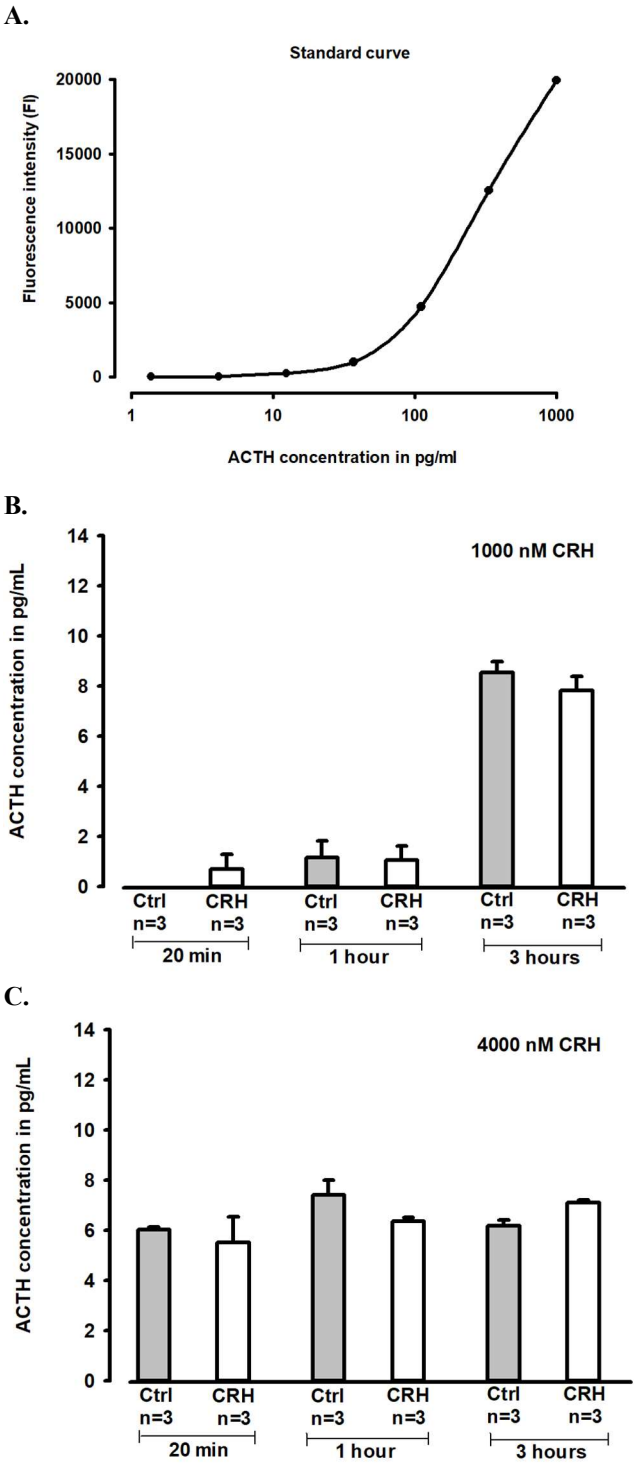


Figure 13. Luminex results. The concentration of ACTH was measured in pg/mL. **A)** The standard curve. The concentration is calculated based on the fluorescence intensity. The formula for the calculation of the standard curve in the software; Std. Curve: FI = -2.27241 + (23632.9 + 2.27241) / ((1 + (Conc / 260.43) ^-1.34955)) ^1.15163.**B)** The concentration of ACTH in samples exposed to 1000 nM CRH and controls. All data are given as the mean ± SEM. **C)** The concentration of ACTH in samples exposed to 4000 nM CRH and controls. All data are given as the mean ± SEM.

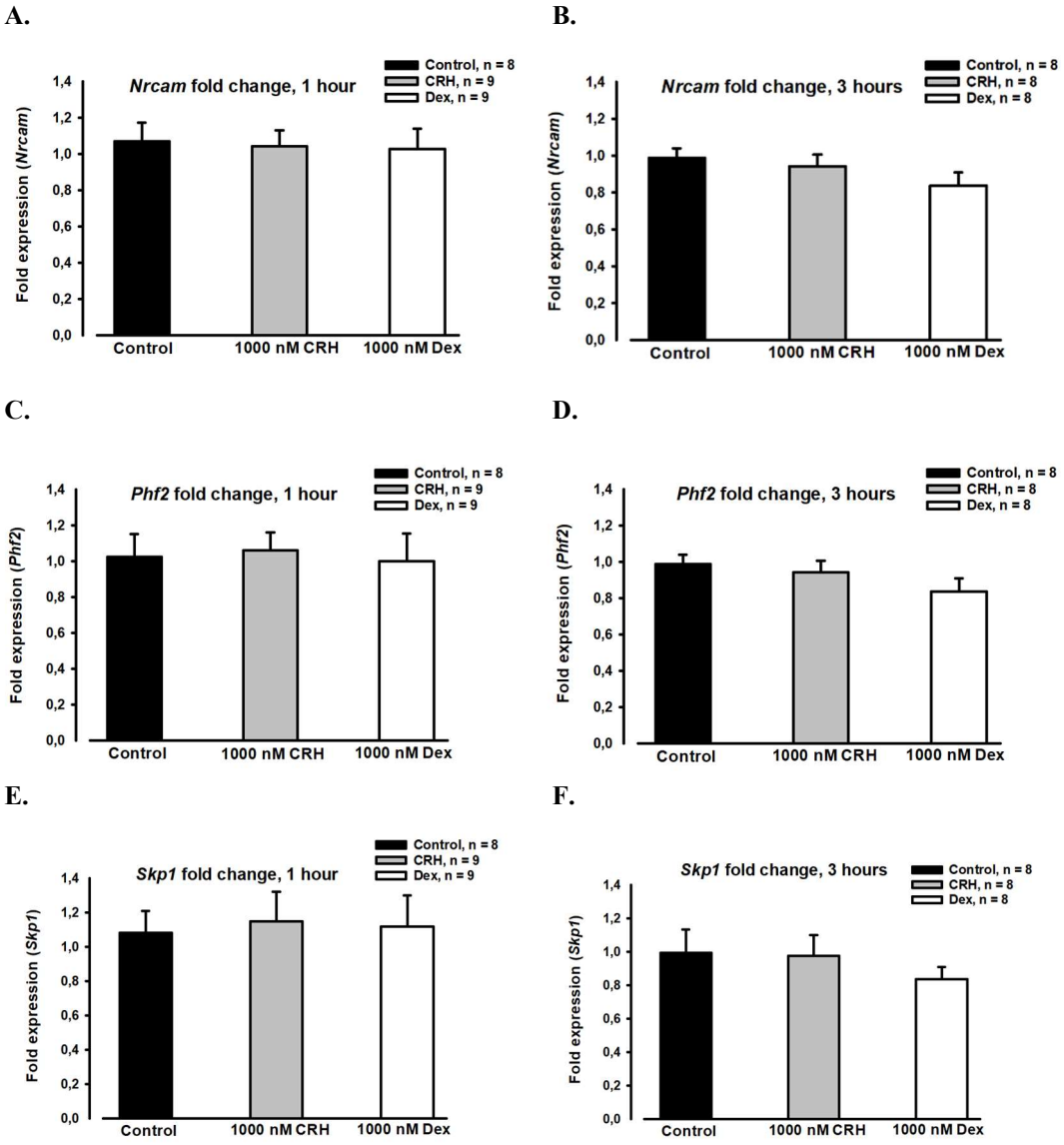


Figure 14. The fold expression of the genes Nrcam, Phf2 and Skp1 for cells exposed to 1000 nM CRH or 1000 nM Dex: left panel 1 hour, right panel 3 hours. A) and B) The Nrcam fold change expression relative to control. **C) and D)** The Phf2 fold change expression relative to control. **E) and F)** The Skp1 fold change expression relative to control. All data are given as the mean ± SEM.

4.2 The Human cohort

4.2.1 Hardy-Weinberg equilibrium

The Hardy-Weinberg equilibrium was calculated for the three SNPs (rs7209436, rs110402, rs242924) separately (Table 4). No deviation from the Hardy Weinberg equilibrium was observed.

4.2.2 Genotyping/Haplotyping

After reconstructing haplotypes with the software PHASE v2.1.1, the haplotypes with the highest frequencies in the sample population was TAT and CGG, with a frequency of 45 and 51 percent, respectively. Thus, the CRHR1 haplotyping revealed three main haplotypes, TAT/TAT, TAT/CGG and CGG/CGG (Table 5). Therefore, only the combinations, TAT/TAT, TAT/CGG and CGG/CGG, were used for the statistical analyses, and the sample population consisted of complete data from 1083 subjects. The other haplotypes were excluded due to low frequency; they constituted 61 out of 1184 samples (Table 6).

4.2.3 Statistics

The results from the statistical analyses are described in the tables 7–10 below. The characteristics of the male and female subjects is presented in table 7 and 8, respectively. There were more female (572, 52.81 percent) than male (511, 47.18 percent) subjects included in the sample population. A higher number of female subjects answered “Yes” when asked if they were bullied in the workplace, compared to males (62 and 48, respectively).

The result of the hierarchical linear regression analyses is described in table 9. The main effect (Step 1) and the two-way interaction (Step 2) between exposure to workplace bullying and the haplotype was assessed. The result of Step 1 demonstrated a significant effect of bullying on symptoms of depression for both males and females, with a p-value of <0.001 for both genders. The beta coefficient values indicated an increase regarding the effect of bullying with 0.332 for male subjects and 0.254 for female subjects. The result for the effect of the CRHR1 haplotype alone on symptoms of depression showed no significant association for either gender.

Step 2 demonstrated a possible two-way interaction between workplace bullying and the different CRHR1 haplotype combinations. Bullying was the only statistically significant variable for males and females (p-values of <0.001). Moreover, the two-way interaction (bullying x haplotype) for the female cohort showed a significant effect when comparing the different haplotype combinations. Using female TAT/TAT carriers as a reference, both TAT/CGG and CGG/CGG carriers showed a significantly reduced effect of bullying (p-values of 0.027 and <0.001, respectively). The beta coefficient was -0.283 for TAT/CGG carriers and -0.669 for CGG/CGG carriers. This implies that the CGG haplotype may have a protective effect in these subjects. Furthermore, having two copies of the CGG allele is more protective than one copy. No such effects were observed in the male cohort.

Finally, the results of the three-way interaction between bullying, CRHR1 haplotypes and gender is presented in table 10. The tree-way interaction analysis was implemented to assess any potential gender difference. Compared to males, female carriers of the CGG/CGG haplotype combination showed a significantly higher protective effect against bullying in the workplace (p-value of 0.003, beta coefficient of -0.487).

The symptoms of depression in males and females are summarized in figure 15 A and B, showing the effect of the CRHR1 haplotype in the subjects that experienced bullying in the workplace. No correlation between the CRHR1 haplotype and symptoms of depression was shown in males, whereas in the female cohort, the correlation was clearer. The column representing the female carriers of the TAT/TAT haplotype reported more symptoms of depression compared to TAT/CGG and CGG/CGG carriers.

Table 4. The Chi square score and p-value for the three SNPs.

SNP	CHI SQUARE SCORE	P-VALUE
RS7209436	0,0063	0.9367
RS110402	0,0039	0.9502
RS242924	0,0308	0.8606

Table 5. The occurrence of the different CRHR1 haplotypes.

HAPLOTYPES	NUMBERS
TAT	1088
TAG	3
TGG	2
CAT	54
CGT	2
CGG	1219
TOTAL	**2368**

Table 6. The different haplotype combinations.

HAPLOTYPE COMBINATIONS	NUMBERS
TAT/TAT	251
TAT/TAG	1
TAT/CAT	25
TAT/CGG	560
TAG/CGG	2
CAT/CGG	29
CGT/CGG	2
CGG/CGG	312
TOTAL	**1184**

Table 7. Characteristics of the male subjects by CRHR1 haplotype (rs7209436, rs110402, rs242924); TAT/TAT, TAT/CGG and CGG/CGG.

	Range	TAT/TAT					TAT/CGG					CGG/CGG					Sum
		N	%	Mean	SEM	Y/N	N	%	Mean	SEM	Y/N	N	%	Mean	SEM	Y/N	
Subjects		101	19.8				258	50.5				152	29.7				511
Depression	1 to 4			1.34	0.03				1.30	0.02				1.34	0.03		
Bullying	Y/N					13/88					20/238					15/137	48/463
Age				45.5	1.06				46.0	0.64				46.2	0.79		
Education																	
High school or lower		54	53.5				118	45.7				65	42.8				
University		47	46.5				140	54.3				87	57.2				

N: number of subjects, SEM: standard error of mean, Y/N: Yes/No

Table 8. Characteristics of the female subjects by CRHR1 haplotype (rs7209436, rs110402, rs242924); TAT/TAT, TAT/CGG and CGG/CGG

	Range	TAT/TAT					TAT/CGG					CGG/CGG					Sum
		N	%	Mean	SEM	Y/N	N	%	Mean	SEM	Y/N	N	%	Mean	SEM	Y/N	
Subjects		141	24.7				279	48.8				152	26.6				572
Depression	1 to 4			1.44	0.03				1.41	0.02				1.37	0.03		
Bullying	Y/N					14/127					38/241					10/142	62/510
Age				43.5	0.81				44.9	0.59				44.5	0.77		
Education																	
High school or lower		50	35.5				93	33.3				44	28.9				
University		91	64.5				186	66.7				108	71.1				

N: number of subjects, SEM: standard error of mean, Y/N: Yes/No

Table 9. Hierarchical regression analysis of the effect of Bullying on depression; main effects and two-way interaction (Bullying^{x}CRHR1) stratified by gender. The analysis was adjusted for age and education. Number of observations = 1.083.

	Men				Women			
	B	**Std. Err.**	**p-value**	**95% CI**	**B**	**Std. Err.**	**p-value**	**95% CI**
Step 1								
Main effects								
Bullying	**0.332**	0.570	**<0.001**	(0.220, 0.444)	**0.254**	0.053	**<0.001**	(0.150, 0.359)
CRHR1 haplotype								
TAT/CGG	-0.019	0.044	0.666	(-0.105, 0.067)	-0.042	0.040	0.298	(-0.122, 0.037)
CGG/CGG	0.014	0.048	0.769	(-0.080, 0.108)	-0.061	0.045	0.184	(-0.082, 0.012)
Step 2								
Bullying (given that CRHR1 = TAT/TAT)	**0.395**	0.111	**<0.001**	(0.176, 0.614)	**0.538**	0.109	**<0.001**	(0.323, 0.753)
CRHR1 haplotype								
TAT/CGG	-0.060	0.046	0.897	(-0.097, 0.085)	-0.014	0.042	0.735	(-0.097, 0.069)
CGG/CGG	0.018	0.051	0.718	(-0.081, 0.118)	-0.007	0.047	0.874	(-0.100, 0.085)
Two-way interaction								
TAT/CGG	-0.127	0.140	0.365	(-0.404, 0.149)	**-0.283**	0.128	**0.027**	(-0.535, -0.031)
CGG/CGG	-0.026	0.150	0.862	(-0.321, 0.269)	**-0.669**	0.167	**<0.001**	(-0.997, -0.340)

B: beta coefficient, Std. Err.: standard error, CI: confidence interval, reference group: TAT/TAT haplotype

Syntax STATA:

Step 1*: by Gender, sort: regress MeanDepression Bullying Age Education i.CRHR1*

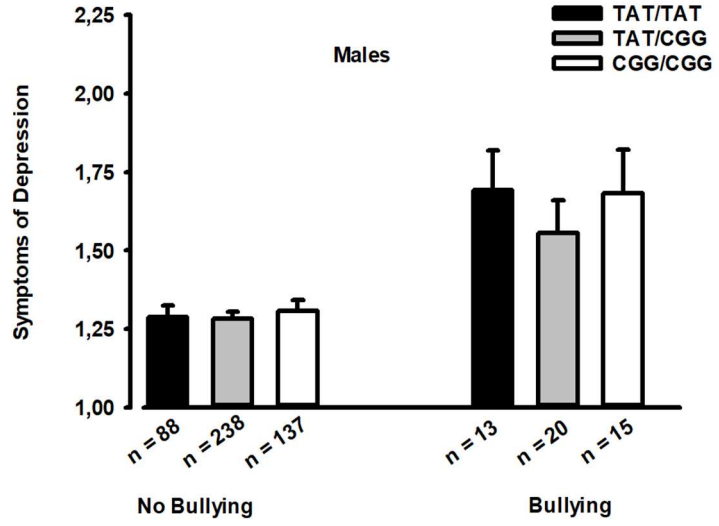
Step 2*: by Gender, sort: regress MeanDepression i.Bullying Age i.CRHR1 Education Bullying#i.CRHR1*

Table 10. Linear regression analysis of the effect of bullying on depression, three-way interaction (Gender^{x}CRHR1^{x}Bullying). The analyses were adjusted for age and education.

	B	Std. Err	p-value	95% CI
CRHR1 - gender difference (reference male)				
Female (TAT/TAT)	0.151	0.156	0.334	(-0.155, 0.457)
Female (TAT/CGG)	-0.009	0.111	0.935	(-0.227, 0.208)
Female (CGG/CGG)	-0.487	0.162	**0.003**	(-0.806, - 0.169)

B: beta coefficient, Std. Err.: standard error, CI: confidence interval
Syntax STATA: *regress MeanDepression Education Age i.Gender i.Bullying i.CRHR1 i.Gender#i.CRHR1 Bullying#i.CRHR1 Bullying#i.CRHR1#i.Gender*

A.



B.

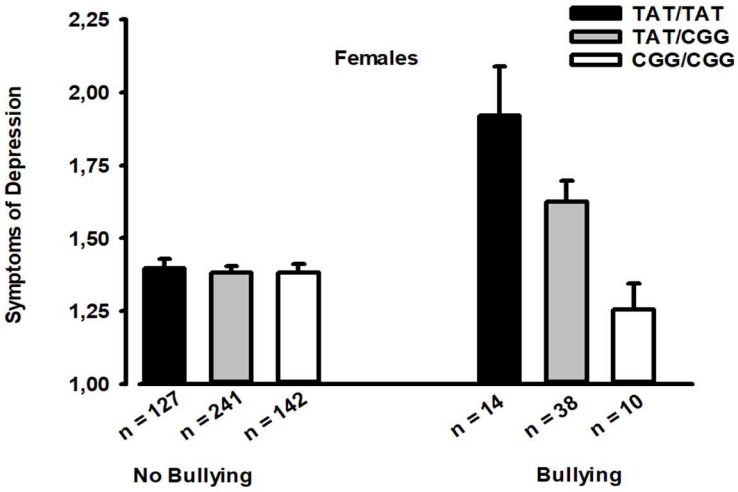


Figure 15. Symptoms of depression in men and females. A) Stratified by the CRHR1 haplotypes (TAT/TAT, TAT/CGG, CGG/CGG) and the number of males who answered "no" and "yes" when asked if they were bullied in the workplace. Adjusted for age and education. **B)** Stratified by the CRHR1 haplotypes (TAT/TAT, TAT/CGG, CGG/CGG) and the number of females who answered "no" and "yes" when asked if they were bullied in the workplace. Adjusted for age and education.

5. Discussion

5.1 The *in vitro* arm

Previous data show that the resident-intruder paradigm may have affected the gene expression of *Pomc, Nrcam,* and *Phf2* in the pituitary glands of the Sprague Dawley rats (section 1.3.1/1.3.2). Therefore, it was hypothesized that the stress hormones corticotropin-releasing hormone (CRH) and Dexamethasone (Dex) could affect gene expression in the RC-4B/C cell line. Based on the knowledge of the hypothalamic-pituitary-adrenal (HPA) axis (section 1.2.), CRH should theoretically lead to an upregulation of *Pomc* (the ACTH precursor) and release of adrenocorticotropic hormone (ACTH) from the corticotrophs in the pituitary cell line.

However, the present thesis did not demonstrate a significant association between exposure to CRH or Dex and the stress response in the RC-4B/C cell line.

5.1.1 Methodological considerations

5.1.1.1 Cell medium composition

An important factor to consider when discussing the results, is the state of the RC-4B/C cells. The cells spent their last 24 hours in an incubator without fetal bovine serum (FBS) in their cell medium due to concerns that FBS present in the media may interfere with downstream analyses [80]. FBS is a vital component to provide growth promoting and survival enhancing factors to the cells, and the deprivation of FBS may affect the gene regulation or cause stress [81, 82]. However, it is difficult to determine the effect of the starvation of FBS on the RC-4B/C cell line, and how much the FBS starvation potentially affected the gene expression pattern after stimulation with CRH or Dex. If the cells were stressed due to lack of FBS, it might affect the stress response we attempted to assess in the cell line. The RC-4B/C cells were only exposed to CRH or Dex for a maximum of three hours, which is a short amount of time compared to the approximately 24 hours they were in a medium without FBS.

5.1.1.2 The number of passages

Another important factor that could potentially have affected the results of the ACTH immunoassay and the RT-qPCR, is the amount of time that passed between the start of the first experiment and the last experiment, i.e. the number of times the cells were subcultured. The first experiment was conducted after passage 12 and the last one after passage 21. There is no guarantee that the cells have not undergone small alterations during this time. Previous studies have shown that cell lines at high passage numbers can be affected in several aspects, such as morphology, response to stimuli and protein expression [83]. This might explain the unaltered ACTH concentration in the cell medium isolated from RC-4B/C cells after exposure to CRH (1000 nM/4000 nM) compared to controls, and the lack of significant changes in the gene expression in the RC-4B/C cells after exposure to CRH (1000 nM/4000 nM) or Dex (1000 nM) compared to controls. However, there is no clear definition of the maximum number of passages a cell line can go through before these changes occur. It depends on type of tissue, cell line organism of origin and other factors [83]. Cell lines are technically immortal; however, it is generally understood that the fitness of the cell will decrease with the number of passages [84].

5.1.1.3 Cell model

There can be several possible reasons for the unaltered ACTH release into the cell medium of the CRH exposed (1000 nM/4000 nM) RC-4B/C cells compared to the control cells.

First, a potential source of error might be the RC-4B/C cell line itself. As mentioned earlier, the RC-4B/C cell line used in the present thesis was isolated from a rat pituitary adenoma many years ago and spent five years in culture prior to cryopreservation. Moreover, according to the researchers responsible for the establishment of the cell line, the RC-4B/C cells displayed a more altered phenotype compared to normal anterior pituitary cells from rats in year five. Furthermore, the researchers also reported the presence of endogenous C-type rat retroviral particles that may infect and transform the secretory epithelial cells in the RC-4B/C cell line [70]. The RC-4B/C cell line contains the ACTH producing gene [70]. However, a search of the literature did not reveal any studies that tested whether the cell line is able to secrete ACTH in response to stimulation with CRH.

Furthermore, the RC-4B/C cell line consist of all the known types of anterior pituitary cell types [71]. This means that the cell line does not consist of corticotrophs exclusively; corticotrophs constitute only approximately ten percent of pituitary cell cultures [70, 85] and the RC-4B/C cell line contains approximately nine percent corticotrophs [70]. The low percentage of ACTH-producing cells in the RC-4B/C cell line might explain the unaltered ACTH release into the cell medium for cells exposed to CRH compared to cell medium from control cells.

A cell line consisting of corticotrophs only, could possibly respond in a different manner after stimulation with CRH, such as the mouse cell line AtT-20, a pituitary corticotroph cell line known to secrete ACTH [86]. The AtT-20 cell line might therefore be a more ideal cell line for the study of stress response in corticotrophs [87], although from a different species than what was used for the resident-intruder paradigm study.

The second possible source of error is also related to the use of the RC-4B/C cell line, concerning the use of a cell line to replicate results derived from studies in rats. The use of cell lines to replicate findings from primary cells was evaluated in a study by van den Brand et al. [88]. The authors compared the use of primary pituitary cells from rats with two pituitary cell lines from rats, GH3 and RC-4B/C, to study the regulation of prolactin. The primary pituitary cells had been isolated directly from rat pituitaries. They showed that the prolactin regulation in the primary pituitary cells *in vitro* was very different from the prolactin regulation in the two cell lines. Additionally, they found that the basal prolactin secretion and gene expression in RC-4B/C cells were lower than in the primary pituitary cells. This might only be relevant for the study of the regulation of prolactin. However, it underlines the importance of evaluating the difference between cells derived directly from the pituitary of rats and those isolated to develop a commercial cell line. Furthermore, the RC-4B/C cell line derives from an anterior pituitary adenoma, a benign tumor. This means that the RC-4B/C cell line derives from cells that differ from normal anterior pituitary cells to begin with.

5.1.1.4 Half-life of ACTH

Another possible source of error could be caused by the half-life of ACTH. The function of ACTH, as described in section 1.2, is to travel through circulation to trigger the synthesis and release of CORT from the adrenal glands. ACTH acts rapidly in the organism, and consequently, the hormone has a short half-life. *In vivo*, the half-life of ACTH in rats is only four minutes [15], whereas evidence suggest that the degradation of ACTH takes longer *in vitro*. ACTH isolated from cell medium from primary cultures and AtT-20 cultures has been detected and harvested after three–six hours [89, 90]. During the experiments conducted in the present thesis, cell medium from RC-4B/C cells was isolated after 20 minutes, one hour and three hours of exposure to CRH (1000 nM/4000 nM). However, the concentration of ACTH was close to the background medium control for isolated cell medium both from CRH exposed cells and from control cells for all the timepoints.

To prevent a potential degradation of ACTH during the exposure experiments, the cell medium was kept cool at four degrees Celsius once it was isolated up until the point where it was placed in a -80 degrees freezer. The cell plates were immediately placed on ice, but they had been kept on incubation at 37 degrees until the medium was isolated. It takes time for the medium to cool down to four degrees and it is unclear how much this temperature change may affect the degradation of ACTH.

5.1.1.5 The immunoassay kit

The MILLIPLEX® kit used for the immunoassay has an accuracy for detection of ACTH at 83 percent, and sensitivity at 0.9 pg/mL [91]. This means that the immunoassay kit can detect ACTH at very low levels. Lack of accuracy and sensitivity in the kit can therefore be ruled out as possible reasons for the lack of detection of ACTH. However, the standard curve is required to cover the range of concentrations measured from the samples. The lowest value of the standard curve is 1.4 pg/mL, any concentrations lower than 1.4 pg/mL cannot be determined.

5.1.2 Discussion of the results

5.1.2.1 ACTH immunoassay

In this thesis, exposure to 1000 nM and 4000 nM CRH, respectively, was tested in the RC-4B/C cell line. The aim was to measure the release of ACTH into the cell medium as a response to exposure. However, many of the samples exposed to CRH (1000 nM/4000 nM) had a concentration that was slightly above the background medium control concentration of ACTH (Figure 13 A + B), and in the same concentration range as the control samples. Both the CRH-exposed and the control samples were in the lower range of the standard curve, and the accuracy of the standard curve is somewhat uncertain in the lower range. Moreover, several of the medium samples tested in the present thesis, both CRH-exposed and controls, had concentrations that were out of range. The maximum concentration measured is the cell medium isolated from CRH-exposed samples and controls was around 10 pg/mL.

The cell media isolated from control cells and the cells exposed to CRH (1000 nM) showed a higher concentration of ACTH after three hours compared to the concentration after 20 minutes and one hour. This pattern of upregulation was not observed in the cells exposed to 4000 nM CRH or the controls. However, the experiment was conducted with a somewhat limited number of replicates, which makes it difficult to determine the cause of the upregulation.

As previously described, the FBS in the cell medium for the RC-4B/C cells was removed approximately 24 hours before the exposure experiments, as FBS starvation is a common technique to prevent interference with the results [80]. However, if the cells were stressed due to lack of FBS, it might have interfered with the investigation of stress response in the cell line.

As a preliminary part of the present thesis, the gene expression levels for the gene *Pomc* were tested. However, the gene expression levels for the gene *Pomc* were not evaluated for RC-4B/C cells after CRH or Dex exposure, due to low levels of expression in this cell line. Testing of a primer for *Pomc* in this cell line, yielded exceedingly high Ct values. The Ct values at 20 ng input of cDNA were around 36, which is the same level as many of the non-template controls, suggesting that the actual gene expression of *Pomc* in the RC-4B/C cells is probably low. Moreover, since the expression of *Pomc* was so low, it was not possible to investigate whether exposure to CRH (1000 nM/4000 nM) or Dex (1000 nM) lead to an up– or downregulation of *Pomc* in the cells. Additionally, the possibly low expression of *Pomc* in the RC-4B/C, the precursor of ACTH (section 1.2), could be a potential explanation for the low ACTH concentration measured in the cell media after exposure to CRH.

Due to a lack of significant results during this first preliminary test, some of the isolated cell media from the cells included in the CRH exposure experiments were not tested for the presence of ACTH.

5.1.2.2 Gene expression of *NRCAM, PHF2 & SKP1*

As described in section 1.2, a release of CRH from the hypothalamus initiates the transcription of *Pomc* and the release of ACTH from corticotrophs in the anterior pituitary. Cortisol and corticosterone (CORT) in humans and rats, respectively, are the hormones that exert the function of the HPA axis for the organism to adapt to the stressor and reestablish homeostasis. The exposure of the RC-4B/C cell line to CRH (1000/4000 nM) or the synthetic CORT, Dex (1000 nM), did not result in any significant changes in the gene expression of *Nrcam, Phf2,* and *Skp1* compared to controls.

The exposure of the RC-4B/C cell line to 1000 nM CRH resulted in small, non-significant, trend towards reduced gene expression of the selected genes *Nrcam, Phf2* and *Skp1* compared to control cells after three hours. A similar trend was not observed after three hours of exposure to 4000 nM CRH, except from a non-significant trend towards reduced gene expression for *Nrcam* compared to control cells. The explanation for the difference between exposure to the two different concentrations of CRH, 1000 and 4000 nM, is unknown. Exposure of 4000 nM of CRH was not cytotoxic; exposure to 4000 nM CRH was less cytotoxic than exposure to 1000 nM CRH, assessed by LDH toxicity assay, see supplementary figure 1. Due to the lack of significant changes in the gene expression levels after exposure to 1000 nM or 4000 nM of CRH it is difficult to assess which or if any concentration of CRH could be more physiologically relevant for the assessment of the stress response in the RC-4B/C cell line.

Exposure to Dex (1000 nM) in the RC-4B/C cell line showed a possible trend towards downregulation in the gene expression of the selected genes *Nrcam, Phf2* and *Skp1* in cells after three hours of exposure, compared to the expression in the controls. When CORTs exert their function in the organism, they affect the regulation and transcription of many genes [19]. These genomic effects of CORTs may result in long-lasting effects, lasting from hours to days [15]. However, these genomic effects may require some time delay before the effects in the cellular function may be observed, previous studies have reported that it may take >60 min [15].

Recent RNA sequencing of the rat pituitary [34] shows that *Nrcam* and *Phf2* are down- or upregulated, respectively, by social stress in vivo. As described above, both genes showed a trend towards downregulation in the RC-4B/C cells after three hours exposure to Dex or CORT, particularly after exposure to Dex. A trend towards downregulation of *Nrcam* was expected based on the RNA-sequencing, however, the downregulation of *Phf2* was not. The NRCAM protein is an important molecule involved in signal transduction and has also been linked to neurons involved in memory [92]. Moreover, stressful stimuli, meditated by CORTs, are known to affect memory [2], and this possible connection might provide an explanation for the association between the stressful stimuli in the Sprague Dawley rats and the downregulation of the *Nrcam* gene.

The reason for the downregulation of *Phf2* in the RC-4B/C cell line after exposure of 1000 nM Dex is unknown. The PHF2 protein, which activates promoter regions for transcription by removing repressive methylation modifications on histone tails [93], should theoretically be upregulated if stimulation with Dex led to a more active transcription pattern in the RC-4B/C cells. However, the effect of Dex exposure on anterior pituitary cells is not known and it might be possible that Dex leads to a less active transcription pattern in the anterior pituitary cells. Moreover, it is important to note that even though the stress-exposed Sprague Dawley rats had an increased expression of *Phf2* in their anterior pituitary gland, the effects on *Phf2* could possibly be mediated by other factors. Since the increased expression may be affected by other factors, potential comparison between cell lines and rats are challenging. It is also important to note the time difference between the exposure experiments in the RC-4B/C cells and the resident-intruder paradigm. The resident-intruder paradigm lasted for seven days before the anterior gland was harvested and the RNA isolated. However, the *in vitro* exposure to Dex in the RC-4B/C cell line lasted for a maximum of three hours. It is not unlikely that the stress-induced gene expression changes observed in the Sprague Dawley rats took more than three hours, as the rats were exposed to stress throughout a week.

The gene expression levels for the *Skp1* gene was also assessed in the RC-4B/C cells, post exposure to stress hormones. The reason for the inclusion of *Skp1* was to investigate if there was a co-expression of the proteins PHF2 and SKP1, that had previously been demonstrated in *Homo sapiens* (section 1.3.3). The same trend towards downregulation can be seen for the *Phf2* and *Skp1* genes after three hours of exposure to 1000 nM Dex (Figure 14 D). However, none of the results after of the gene expression analyses of *Phf2* and *Skp1* were significant, and thus no conclusion should be made regarding the potential co-expression of SKP1 and PHF2 in the RC-4B/C cell line.

5.1.2.3 The use of the RC-4B/C cell line to study the stress response

The RC-4B/C cell line was chosen for the assessment of the stress response in the present thesis because it was the only available cell line derived from a rat anterior pituitary. As previously described (section 1.2) the anterior pituitary is central in the HPA axis response to stressors due to the corticotrophs. Because one of the aims in the present study was to further examine the previously described stress-induced altered gene expression found in stressed Sprague Dawley rats, it was decided to investigate potential changes in gene expression in a cell line from the same organism. However, the results after the gene expression analyses of the genes *Nrcam, Phf2* and *Skp1* showed no significant changes.

The use of the RC-4B/C cell line to study the stress response was not successful. As discussed, the theoretical effect of CRH should have been an increased expression of *Pomc* and secretion of ACTH. Any potential effect on the expression of *Pomc* could not be evaluated due to the unsuccessful primer testing. In addition, the ACTH release into the CRH-exposed cells were unaltered compared to the medium from control cells.

The RC-4B/C cell line has a heterogenous cell population [70], and the presence of other cell types may be the reason why the results differ from expected results. In a previous study that investigated the responsiveness of the RC-4B/C cell line to the hormone ghrelin [94], found that the RC-4B/C cells responded to ghrelin. However, a further investigation into ghrelin signaling in the cells was not successful. The authors suggested that the further characterization of the ghrelin signaling pathway was complicated by the presence of other cell types in the RC-4B/C cell line. The ghrelin pathway occurs in the growth-hormone producing cells in the anterior pituitary, which makes the target cells for that study different than the target cells studied in the present thesis. However, it is interesting to note that the authors also reported diminished ghrelin responsiveness after only ten culture passages [94].

Although the use of animal models to study the stress response may provide greater insights into the stress response affecting the entire animal, a cell line model represents an opportunity to study the effects of stress in more detail, and to assess how the stress response affects one organ or cell type. This method may provide greater insights into how each component in the HPA axis responds to stress separately, instead of the stress response in the organism. Moreover, the use of a cell line as a model for the stress response is both less ethically problematic [95] and more cost-effective [96].

5.2 The Human arm

A human cohort was used to assess social stress in humans in the form of workplace bullying which has previously shown to have adverse consequences in the form of various psychiatric disorders, such as depression and anxiety [48]. The aim in the present thesis was to assess if single nucleotide polymorphisms located in the *CRHR1* gene, which encodes an important receptor in the HPA axis, could moderate the outcome, i.e. symptoms of depression, when the subjects were exposed to workplace bullying.

The present thesis demonstrated a possible association between workplace bullying, symptoms of depression, and the CGG/CGG haplotype in female carriers. Female carriers of the CGG/CGG haplotype who had been exposed workplace bullying showed reduced symptoms of depression compared to female carriers of the TAT/TAT and TAT/CGG haplotypes (Table 9–10). These results were not replicated in males.

5.2.1 Methodological considerations

The result from the haplotyping (section 4.2.2) showed that most of the subjects in the human cohort had the haplotype TAT or CGG, or a combination of those two haplotypes; the subjects were either homozygote or heterozygote carriers. However, it is important to note that the Phase software reconstructs haplotypes based on a statistical estimation. The statistical estimation is based on the frequencies of the preexisting genotypes in the sample population. This means that any individual with an undetermined haplotype was assigned a haplotype based on preexisting frequencies in the sample population [76]. This is a limitation of the method used in the present thesis. However, the only way to get a dataset that is 100 percent accurate is to perform DNA sequencing on the saliva samples from the human cohort. DNA sequencing is a much more time consuming and expensive method to evaluate polymorphisms in a population. Therefore, the current method for haplotyping was accepted, despite its limitations.

Another factor that may affect the results is human error. When handling large datasets, it can be difficult to detect small irregularities that may affect further analyses. Any potential errors might have occurred during several steps: the initial processing of the questionnaire, the isolation of genomic DNA, the genotyping, haplotyping, or during the statistical analyses.

5.2.2 Discussion of the results

5.2.2.1 SNPs in the intronic region

An important question regarding the role of the SNPs in the *CRHR1* gene investigated in the present thesis is how the SNPs possibly function as moderators when located in the intronic regions. It has been proposed that the SNPs investigated in the present thesis are not the functional variants [61], and might be found in linkage disequilibrium with a functional SNP that has yet to be discovered. However, previous findings suggest that intronic SNPs can affect gene function by altering transcriptional regulation [97, 60], gene-splicing, promoter activity, and the efficacy of transcription and translation [98, 99].

Previous studies have shown that SNPs located in genes involved in stress response were associated with the different coping mechanisms [100] and an altered HPA axis activity [101]. Moreover, SNPs in the *CRHR1* gene different from the SNPs investigated in the present thesis have been associated with suicidality [102] and panic disorder [103].

A study by Labermeier et al. [104] investigated how an SNP located in intron 1 in the *Crhr1* gene in male mice affected the corticosterone (CORT) levels after exposure to chronic stress. The selected SNP functioned as a tag for other haplotypes. The authors discovered that TT homozygotes had a higher level of *Crhr1* mRNA compared to CC homozygotes, and consequently an increase in expression of CRHR1 in several areas of the brain, including the anterior pituitary. The mice with increased CRHR1 expression also showed increased levels of CORT following stress exposure. The authors could not identify which of the 109 SNPs that were in strong linkage disequilibrium with the tag SNP that led to the actual upregulation of CRHR1. However, several of the SNPs were predicted to affect the function in various ways, e.g. transcription factor binding. The authors therefore proposed that the SNPs are involved in the transcriptional regulation of *Crhr1*.

It is important to further investigate how the SNPs in the present thesis (rs7209436, rs110402, rs242924) possibly affect the function of *CRHR1* to determine if the association between the SNPs and the outcome (symptoms of depression) is a real consequence of the SNPs, or an arbitrary association. The SNP rs110402 maps to a DNase I hypersensitivity site in the brain [104], which are sites associated with accessible chromatin [105] and have previously been associated with various diseases [106, 104]. The location of the SNP rs110402 might provide a possible explanation for how the SNPs affect the function, by affecting the transcription of *CRHR1*.

5.2.2.2 The potential protective effect of the TAT haplotype

As described in the introduction, previous studies have shown an association between the TAT haplotype and a protective effect against depression in subjects exposed to early life stress. However, other studies have also found an association between the TAT haplotype and a greater risk of depressive symptoms and deteriorating cognitive function in patients with MDD. The results of the haplotyping and the subsequent statistical analyses in the present thesis demonstrated that female carriers of the haplotype CGG/CGG were less affected by bullying and thereby exhibited a protective effect against symptoms of depression (Tables 9 and 10). Furthermore, female carriers of only one copy of the CGG haplotype showed a protective effect compared to those with two copies of the TAT haplotype.

This contradicts the findings in the studies by Bradley et al., Tyrka et al. and partially the studies by Polanczyk et al. and Kranzler et. al. [61, 65, 62, 58] whose findings supported the potential protective effect of the TAT haplotype. The results in the present thesis are similar to the findings by Grabe et al. [66], Davis et al. [67] and partially the study by Kranzler et al.; where the TAT haplotype was associated with a greater risk of depressive symptoms [66], a worse cognitive effect in patients with MDD with more copies of the TAT haplotype [67], and a 50 percent greater chance of developing MDD with two copies of the TAT haplotype if the subject had no previous experience of childhood trauma [58].

5.2.2.3 The exposure difference

Furthermore, when comparing the results from the present thesis with the studies that found an association between the TAT haplotype and reduced symptoms of depression/less MDD it is also important to compare the stress exposure in the different sample populations. The subjects in the studies by Bradley et al., Tyrka et al., Polanczyk et al. and partially the study by Kranzler et. al. [61, 65, 62, 58] reported past exposure of childhood trauma. The subjects who were carriers of the TAT haplotype in those studies were only protected against MDD/symptoms of depression when previously exposed to early life stress. However, the questionnaire utilized in the present thesis did not include any questions regarding the subject's childhood. Therefore, a fair comparison cannot be made between the subjects in the present thesis and those in the studies by Bradley et al., Polanczyk et al., Tyrka et al. and partially by Kranzler et al.

Early life stress and genetic predisposition may affect the stress reactivity in adults [18], where the response to long-lasting stressors may be modulated by the history of the individual in combination with a genetic predisposition. Early life stress in the form of childhood maltreatment may predispose individuals to depression as adults due to an increased HPA axis reactivity [68].

Based on the studies by Bradley et al., Tyrka et al., Polanczyk et al. and partially the study by Kranzler et. al. [61, 65, 62, 58], it is possible that the TAT haplotype might confer some form of resilience against depression in adults when exposed to early life stress. However, the study by Grabe et al. [66] also assessed the effect of the TAT haplotype in subjects previously exposed to childhood trauma and found an association between the TAT haplotype and increased symptoms of depression. Because of this contradicting study, it is difficult to determine if the potentially protective effect of the TAT haplotype depends on the subject's previous exposure to early life stress. For the present thesis, it is important to consider the difference between the sample populations with regards to the type of stress exposure.

5.2.2.4 The ethnicity of the sample population

Another important factor to consider is the ethnicity of the subjects that were included in the studies discussed in the present thesis. The hypothesis regarding the protective effect of the TAT haplotype has been tested on subjects from different ethnic groups. Bradley et al. [61] investigated the hypothesis regarding the TAT haplotype in subjects from the African American population and replicated the findings in a smaller sample population consisting of Caucasian women. The study by Kranzler et al. [58] only found a correlation between the TAT haplotype and major depressive episodes in African American women, despite the inclusion of African American men and European American women and men in the sample population. The study by Tyrka et al. [62] tested the association between HPA axis reactivity and two of the SNPs included in the present thesis in a Caucasian sample population. The study by Polanczyk et al. [65] had two predominantly Caucasian sample populations. One may therefore claim that the suggested protective effect of the TAT haplotype has been substantiated in subjects from different ethnicities. The two articles that discussed the adverse effects of the TAT haplotype, by Grabe et al. [66] and Davies et al. [67], assessed Caucasian subjects only. The subjects in the human cohort in the present thesis were also predominantly Caucasian.

The frequency of the two haplotypes, CGG and TAT, did not deviate much from each other between the discussed studies and the present thesis. The CGG haplotype consisted of the major alleles, and the TAT haplotype of the minor alleles, for the studies that reported the haplotype frequencies. The frequency of the haplotypes in the present thesis was 51 and 45 percent for CGG and TAT, respectively. The frequency for the two haplotypes was not provided in all the studies discussed in the present thesis. However, Polanczyk et al. [65] reported a percentage frequency of approximately 56 (CGG) and 42 (TAT) in their two Caucasian sample populations. Kranzler et al. [58] reported a percentage frequency of 67.3 (CGG) and 27.9 (TAT) in their African American sample population and 54.3 (CGG) and 41 (TAT) in their European American sample population. Bradley et al. [61] reported a percentage frequency of 66.5 (CGG) and 28.8 (TAT) in their African American sample population, and 56 (CGG) and 41.6 (TAT) in their smaller Caucasian sample population.

Although the TAT haplotype consists of the of the minor alleles for the different sample populations, the frequency of TAT in the Caucasian sample populations appears to be higher than in the African American sample populations.

5.2.2.5 The gender difference in the sample population

Previous studies have found that females have higher occurrence of major depression and more symptoms of depression compared to males [107]. The females included in the statistical analyses in the present thesis reported on average of more symptoms of depression compared to males, this applied for both females who reported bullying and those who did not (Tables 7 + 8, Figure 15). Various reasons have been implicated as potential causes for this gender difference regarding symptoms of depression, such as females' tendency to use rumination as a coping style more than males when exposed to stressors. Previous studies have shown a link between rumination, as a coping style for stress, and depression [108].

5.2.2.6 Size of sample population

In the present thesis, the total sample population with the TAT/TAT, TAT/CGG and CGG/CGG haplotypes consisted of 1123 subjects. However, the subjects that reported bullying in the workplace consisted only of 48 men and 62 women. One of the reasons for the low frequency of bullying could be that the sample population was selected randomly from the general Norwegian workforce. As the sample population was not included based on predetermined factors such as bullying, there was a risk of low frequency. Another factor that may also have contributed to the low frequency is the low prevalence of workplace bullying in Norway and Scandinavia [109]. An ideal study would include two large sample populations, one that reports bullying and one with no bullying in the workplace. The sample size is too small to draw any conclusions, but it indicates an association between subject's exposure to workplace bullying, the CRHR1 haplotype and symptoms of depression. These results warrant further investigation before any conclusions can be made.

5.2.2.7 The validity of self-reported bullying as a variable

In the present thesis, bullying in the workplace was measured by a single self-report question. Therefore, it cannot be considered as an objective measure since it is the subjects themselves that report the bullying. Additionally, people may have different definitions of bullying, which opens for individual interpretation. This means that in a given situation one person might experience bullying whereas another person might not. It may also depend on the subject's mood on the day they are answering the questionnaire. If they are feeling more vulnerable, they might have a lower threshold for self-reporting as bullied. Another method for measuring workplace bullying is the Negative Acts Questionnaire-Revised (NAQ-R). This tool can be considered a more objective way of measuring negative behaviors in the workplace, which may be described as bullying [110]. The NAQ-R consists of 9 questions and was also included in the questionnaire distributed to the participants. However, statistical analyses with regards to the NAQ-R were not the focus of this thesis.

Self-reported measurement tools will, to a certain degree be, influenced e.g. by factors such as past experiences and the subjects' mood that day. It is possible that some subjects describe themselves as bullied even when they have a low score on for example the NAQ-R. This means that depending on the measurement tools utilized the results, and thus the findings, will differ. A study by Rosander et al. [111] investigated if there was a gender difference between self-reported bullying and NAQ-R. They found that women showed a slightly higher tendency, although not significantly, to self-report as bullied. The most significant finding in the article was that a higher proportion of men could be labelled as bullied based on their answer in the NAQ-R but did not report the experience of being bullied.

Previous studies have shown that self-report of exposure to bullying may be affected by the definition that the subjects have of bullying. A study by Birkeland Nielsen et al. [112] showed that self-report of bullying without a given definition of bullying had a prevalence rate of 18.1 percent, whereas self-report of bullying given a definition was slightly lower (11.3 percent). Self-report studies of bullying without a definition leads to a broad and somewhat unclear definition of bullying, which may unintendedly alter the prevalence rates. Moreover, this implies that the prevalence of bullying in this thesis could have been higher if no definition were included. The questionnaire used in the present thesis included a definition of bullying (see section 3.2.3.1).

5.2.2.8 The use of a human cohort to study biological mechanisms

There are several advantages in assessing genetic polymorphisms in humans. When using cost-effective methods such as genotyping one can attempt to gain a greater insight into potential underlying genetic factors that may moderate the outcome of various stressors. Genetic polymorphisms have previously been associated with both increased and decreased risk of affective disorders [18]. In the present thesis, the potential protective effect of the CGG/CGG haplotype was demonstrated only in female carriers of the haplotype. However, it is difficult to determine if this discovery represents a biological truth, or if men, as discussed in the article by Rosander et al. [111], underreport as bullied and therefore interfere with the statistical analyses. Nonetheless, genotyping of humans may provide interesting insights into biological mechanisms that may moderate the response to external stressors.

6. Future perspectives

One purpose of this master's thesis was to assess if it was possible to study a stress response in the anterior pituitary using the RC-4B/C rat cell line. The gene expression levels in the cells after exposure to 1000 nM of the stress hormones CRH or Dex showed a trend of downregulation after three hours. However, this downregulation was not significant. The cell media isolated from the exposed cells showed ACTH concentrations close to the background medium control. Therefore, it is possible that the RC-4B/C cell line is not the right choice for the study of stress response by any means and that other options, such as the AtT-20 mouse corticotroph cell line, is a better model system for investigation of the pituitary stress response.

7. Conclusion

The overall purpose of this thesis was to investigate the physiological responses induced by stress. First, the effect of stress hormones CRH and Dex in a rat pituitary cell line was investigated. Next, the effect of social stress and genetic variability in the *CRHR1* gene in a human cohort was investigated. Based on the results from this master's thesis, we conclude that:

1a. The result of the ACTH immunoassay after stimulation of the RC-4B/C cells with 1000 nM or 4000 nM CRH, did not indicate any release of ACTH in cell media compared to control cells. However, previous data suggest low levels of *Pomc* in RC-4B/C cells. Thus, the present observation that the ACTH level was in many cases below the detection level may be related to a low ACTH expression in the cell line used in the present study. This may suggest that the RC-4B/C cell line was not an appropriate model for this study.

Ib. Exposure of the RC-4B/C cells with 1000 nM CRH or 1000 nM Dex seemed to be associated with a non-significant possible trend towards downregulation of *Nrcam, Phf2 and Skp1* after three hours. There were no significant changes in the gene expression after exposure with CRH (1000 nM or 4000 nM) or Dex (1000 nM). Whether or not this may be explained by the fact that corticotrophs only constitute small portion of our cell line, remains to be investigated.

II. The data from the human cohort demonstrated that female carriers of the *CRHR1* haplotype CGG/CGG may have a protective effect against the adverse consequences of social stress, such as bullying. However, any potential conclusions regarding the effect of polymorphisms, should be made with caution due to the difficulty in determining causative factors. Moreover, the mechanisms underlying this finding is still poorly understood.

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9. Appendices

Appendix A - RC-4B/C cell culture medium and subculturing of cells

Cells: RC-4B/C, rat pituitary adenoma cells from *Rattus norvegicus* (CRL-1903™, ATCC ®)

Incubation: 37ºC, 5% CO_2.

Table 11. The components of the cell medium (without FBS) for the RC-4B/C cell line.

COMPOSITION	VOLUME	STOCK SOLUTION	PRODUCT NO.
45% DMEM	225 mL	1000 mL	GE Hyclone SH100022.02
45% ALPHA-MEM	225 mL	500 mL	Gibco 22571-020
1% PENICILLIN-STREPTOMYCIN (P/S)	4.5 mL	100x	GE Hyclone SV100010
1X NEAA (NON-ESSENTIAL AMINO ACIDS)	4.5 mL	100x	GE Hyclone E293491000
15 MM HEPES	6.8 mL	1 M	Gibco 15360-056
0.2 MG/ML BSA	2.3 mL	40 mg/mL	GE Hyclone SH100574.01
2.5 NG/ML EGF	450 µL	2.5 µg/mL	Sigma SRP3238-100µg

Complete medium:

45 mL medium + 5 mL dialyzed and heat inactivated FBS (Gibco A33820-01).

Medium change:

1. Prepare complete medium and heat to 37ºC in a water bath.
2. Remove medium from cell culture flask.
3. Add fresh complete medium (25 mL for 75 cm^2 cell flask).

Protocol for subculturing (volumes given for 75 cm^2 cell culture flask):

1. Prepare complete medium.
2. Heat complete medium and trypsin-EDTA (T/E) to 37ºC in a water bath.
3. Remove medium from cell culture flask.
4. Add 2.5 mL T/E to the cell culture flask, swirl over the cell layer and remove the T/E.
5. Add another 2.5 mL T/E to the cell culture flask and place the cells in the incubator for 7 min (37ºC, 5 % CO2).
6. Check the cells in the microscope. If they have not detached, place in the incubator for an additional 5 minutes.
7. Add 2.5 mL complete medium. Resuspend cells by pipetting up and down 3 x 3 times.
8. Check in microscope for aggregates (should be single-cell suspension). Resuspend more if necessary.
9. Add another 2.5 mL complete medium.
10. Count cells on the cell counter (ChemoMeter, NucleoView NC-200).
11. Add the appropriate amount of cell suspension to a new cell culture flask (flasks can be reused one time).
12. Add complete medium to a total volume of 25 mL.
13. Incubate cells at 37ºC, 5% CO_2.

Appendix B - LDH cytotoxicity assay

Kit: CyQUANT LDH Cytotoxicity Assay Kit, Invitrogen (Cat.no. C201000).

Before start:

1. Warm the lysis buffer and stop solution to room temperature (RT).
2. Prepare substrate stock solution: Add 11.4 mL of diH_2O to the entire contents of the substrate mix, then mix gently to dissolve.
3. Prepare assay buffer stock solution: Thaw the assay buffer to RT and protect from light.
4. Prepare reaction mixture: Combine the 600 µL of assay buffer stock solution with the 11.4 mL of substrate stock solution, then mix gently and protect from light until use. Note: Unused reaction mixture can be stored at -20ºC protected from light for 3–4 weeks with tolerance for three freeze/thaw cycles without affecting the activity during the storage period.
5. Prepare 1x LDH Positive control by diluting 1.5 µL LDH positive control with 1 mL of 1 % BSA in PBS. Store the unused portion at – 20 ^{0}C.

Perform chemical compound-mediated cytotoxicity assay:

1. Plate 250 000 cells per well (1 mL medium per well) in three 12-well plates. Incubate at 37ºC, 5% CO_2 for 48 hours before starting the cytotoxicity assay protocol.
2. Change the medium in the wells after approximately 27 hours of incubation. Replace the complete medium with medium without FBS.

Cytotoxicity assay:

1. Starting point (3 hours before harvesting):
    - Add 20 µL of $5*10^{-5}$ M CRH to the “CRH 1000 nM 3 hours well”
    - Add 20 µL of $2*10^{-4}$ M CRH in the “CRH 4000 nM 3 hours well”
    - Add 20 µL of $5*10^{-5}$ M Dex to “Dex 1000 nM 3 hours” wells.
    - Add 20 µL of sterile water to control wells.
2. After 2.15 hours:
    - Add 100 µL of 10x LDH lysis buffer to “maximum” wells.
3. Incubate the plate at 37 ^{0}C, 5% CO_2 for 45 min.

4. After 3 hours (45 min after last addition):
   - Collect all the medium from each well in tubes.
   - Centrifuge for 5 min at 300 x g, RT, to pellet cell debris.
5. Transfer supernatant to new tubes. Transfer 50 µL supernatant from each tube into a clear flat bottom 96-wells plate in duplicate wells.
6. To perform an LDH positive control assay, aliquot 50 µL of 1x LDH positive control into triplicate wells. Aliquot 50 µL of serum-free medium (SFM) into triplicate wells to correct for the endogenous LDH activity present in serum (causes background signal).
7. Add 50 µL of reaction mixture to each sample well. Mix by gentle tapping. Avoid creating bubbles.
8. Incubate the plate at RT for 30 min protected from light.
9. Add 50 µL of stop solution to each sample well. Mix by gentle tapping.
10. Measure the fluorescence by at 490 nm and 680 nm. To determine LDH activity, subtract the 680-nm absorbance value (background signal from instrument) from the 490-nm absorbance value before calculation of % cytotoxicity.
11. Calculate % cytotoxicity by using the following formula:

$$\%\ \text{cytotoxicity} = \left(\frac{\text{Compound}-\text{trea} \quad \text{LDH activity} - \text{spontaneous LDH activity}}{\text{Maximum LDH activity}-\text{Spontaneous LDH activity}}\right) x\ 100$$

Appendix C - Protocol for exposure experiment

Reagents:

- CRH (Cat.no. C3042, Sigma-Aldrich)
- Dexamethasone (Cat.no. D4902, Sigma-Aldrich)

Seeding out – *day 1*

1. Seed out 600 000 cells/well in 6-well plates (1 mL medium per well).
2. Incubate the plates at 37°C, 5% CO_2 for 48 h.

Medium change – *day 2*

Change the cell medium after approximately 24 hours. Replace complete medium with medium without FBS.

Exposure experiment – *day 3*

- Add 20 µL of 5*10-5 M CRH to cells exposed to 1000 nM CRH.
- Add 20 µL of 5*10-5 M Dex to cells exposed to 1000 nM Dex.
- Add 20 µL of $2*10^{-4}$ M to the cells exposed to 4000 nM CRH.
- Add 20 µL of sterile water to the control cells.
- Incubate the plates at 37ºC, 5 % CO_2 until collection of cell medium and/or cells.

Collect medium and harvest cells – *day 3, after 20 minutes, 1 hour and 3 hours*

1. Take the cell culture plate out of the incubator and put on ice immediately.
2. Transfer the medium to 1.5 mL microtubes, put on ice.
3. Spin down medium at 300 x g, 5 min, 4°C to remove cell debris.
4. Transfer the supernatant to new microtubes for later analysis of ACTH level.
5. Store the supernatant at – 80°C.
6. Add 300 µL lysis buffer (SKP buffer from RNA/DNA isolation Norgen kit) to each well. (Note: only isolated cells after 1 hour and 3 hours).
7. Use a cell scraper to detach all cells.
8. Transfer lysed cells to microtubes for later RNA isolation.
9. Store the cells at – 80°C.

Appendix D - Protocol for RNA isolation

Kit: RNA/DNA Purification Kit from Norgen®. (Cat.no. 48700)

Prior to the RNA isolation, the RC-4B/C cells were lysed in the plates with Buffer SKP from the Norgen Biotek RNA/DNA purification kit. The lysis step is described in Appendix C.

Followed the protocol for RNA isolation from Norgen Biotek that was included in Norgen's RNA/DNA Purification Kit.

Reagents:

- Prepare a working concentration of Wash solution A by following the procedure described in the kit protocol. Mix concentrated 38 ml Wash solution A with 90 mL of 96–100 % ethanol in the Wash solution A bottle, giving a final volume of 128 mL.
- All the reagents required in the protocol, except from 96–100% ethanol, were included in the kit.

Genomic DNA purification from the lysate

1. Binding DNA to gDNA Purification Column

a. Assemble a gDNA Purification Column with one of the provided collection tubes. Vortex and centrifuge the lysed cells quickly before loading the cell lysate onto the gDNA Purification Column.

b. Apply up to 600 µL of the lysate onto the column and centrifuge at 5,200 x *g* (~8,000 RPM) for 2 minutes.

c. Retain the flowthrough for RNA Purification. The flowthrough contains the RNA, store on ice until the RNA Purification protocol is carried out.

Total RNA Purification from Lysate

1. Binding RNA to Column

a. Add 60 µL of 96 – 100 % Ethanol to every 100 µL of flowthrough from Step 1c. Mix by vortexing. For example, for 300 µL of flowthrough, add 180 µL of 96 – 100 % Ethanol.
b. Assemble an RNA Purification Column with a collection tube.
c. Apply up to 600 µL of the lysate with the ethanol onto the column and centrifuge at ≥ 3,500 x g (~6,000 RPM) for 2 minutes.
d. Discard the flowthrough. Reassemble the spin column with the collection tube.

2. RNA Wash

a. Apply 400 µL of Wash Solution A to the column and centrifuge at ≥ 3,500 x g (~6,000 RPM) for 1 minute.
b. Discard the flowthrough and reassemble the column with the collection tube.
c. Repeat steps a and b two additional times.
d. Centrifuge the column at 14,000 x *g* (~14,000 RPM) for 2 minutes to thoroughly dry the resin. Discard the collection tube.

3. RNA Elution

a. Place the column into a fresh 1.7 mL Elution tube.
b. Add 50 µL of Elution Solution A to the column.
c. Centrifuge for 2 minutes at 200 x *g* (~2,000 RPM), followed by 1 minute at 14,000 x *g* (~14,000 RPM).

4. Store the purified RNA sample at -80°C.

Appendix E - Protocol for cDNA synthesis

Kit: cDNA synthesis kit from qScript® (Cat.no. 95047-100).

Reagents:

- qScript Reaction Mix (5X). 5X concentrated solution of optimized buffer, magnesium, oligo (dT) and random primers, and dNTPs.
- qScript Reverse Transcriptase, 20X concentration.
- Nuclease-free water

cDNA synthesis protocol:

1. Thaw all frozen reagents. Mix thoroughly and centrifuge briefly to collect contents before using. Place all reagents, including qScript RT, on ice.
2. Prepare a master mix with water, qScript Reaction Mix and qScript RT. Calculate the volume needed of the different reagents in the master mix based on how many wells the master mix will be applied to.

Table 12. Reagents in the master mix that were added to each well during the cDNA synthesis.

REAGENTS	VOLUME IN A SINGLE WELL (µL)
NUCLEASE-FREE WATER	5 µL
QSCRIPT REACTION MIX (5X)	4 µL
QSCRIPT RT	1 µL
TOTAL VOLUME	10 µL

3. Add the following to a 96-well PCR reaction plate sitting on ice:

Table 13. The components in the wells.

REAGENTS	VOLUME (µL)
RNA	Variable
NUCLEASE-FREE WATER	Variable
QSCRIPT REACTION MIX (5X)	4.0 µL
QSCRIPT RT	1.0 µL
TOTAL VOLUME	20.0 µL

4. Add 10 µL RNA sample to each reaction. Dispense 10 µL of cDNA master mix to each well. The RNA was diluted beforehand to a concentration corresponding to a final input of 500 ng and 200 ng RNA into the cDNA synthesis reaction.

5. Cover the reaction plate with sealing film. Vortex gently, and then centrifuge for 10s to collect contents.

6. Place the 96-well plate in a thermal cycler programmed as follows:

1 cycle: 22°C, 5 min

1 cycle: 42°C, 30 min

1 cycle: 85°C, 5 min

4°C hold

Appendix F - Protocol for RT-qPCR

Reagents:

- PerfeCTa SYBR Green FastMix Low ROX (QuantaBio #95074).
- Primers for the genes *Nrcam, Phf2, Skp1* were from Sigma-Aldrich.
- The primer for the endogenous control gene, *Gapdh*, was from Bio-Rad.

All reagents and samples were kept on ice unless specified otherwise.

Protocol for primer testing with standard curves:

1. Master mix for the primers (Table 14 + 15):

Table 14. Overview of contents in the master mix for the primers *Nrcam, Phf2 and Skp1.*

REAGENT	VOLUME/SAMPLE
DDH_2O	0.76 µL
PERFECTA SYBR GREEN FASTMIX	5.00 µL
PRIMER FORWARD (25 PMOL/ µL)	0.12 µL
PRIMER REVERSE (25 PMOL/ µL)	0.12 µL
TOTAL	6 µL

Table 15. Overview of contents in the master mix for the primer *Gapdh*.

REAGENT	VOLUME/SAMPLE
DDH_2O	0.5 µL
PERFECTA SYBR GREEN FASTMIX	5.00 µL
PRIMER (20X SOLUTION)	0.5 µL
TOTAL	6 µL

2. Mix and dilute 40 µL from 20 different cDNA samples (25 ng/µL) to get a stock cDNA solution with a concentration of 5 ng/µL. Prepare a cDNA dilution series to generate a standard curve for each gene, table 16. Test the primer *Gapdh* with the same cDNA dilution series as the other primers, in addition to a cDNA dilution series diluted further 1:10, see table 17.

Table 16. Dilution series for creating the standard curves for primer testing.

STANDARDS	INPUT INTO THE REACTION	CONCENTRATION	VOLUME CDNA	VOLUME H_2O
STD. 1	20 ng	5 ng/µL	200 µL	0 µL
STD. 2	10 ng	2,5 ng/µL	100 µL from Std.1	100 µL
STD. 3	5 ng	1,25 ng/µL	100 µL from Std.2	100 µL
STD. 4	2,5 ng	0,625 ng/µL	100 µL from Std.3	100 µL
STD. 5	1,25 ng	0,313 ng/µL	100 µL from Std.4	100 µL
STD. 6	0,625 ng	0,156 ng/µL	100 µL from Std.5	100 µL
NTC			0 µL	100 µL

Table 17. The 1:10 Dilution series for creating the standard curves for testing of the *Gapdh* primer.

STANDARDS 1:10	INPUT INTO THE REACTION	CONCENTRATION	VOLUME CDNA	VOLUME H_2O
STD. 1*	2 ng	0.5 ng/µL	6 µL from Std. 1	54 µL
STD. 2*	1 ng	0.25 ng/µL	30 µL from Std.1*	30 µL
STD. 3*	0,5 ng	0.125 ng/µL	30 µL from Std.2*	30 µL
STD. 4*	0.25 ng	0,0625 ng/µL	30 µL from Std.3*	30 µL
STD. 5*	0.125 ng	0,0313 ng/µL	30 µL from Std.4*	30 µL
STD. 6*	0,0625 ng	0,0156 ng/µL	30 µL from Std.5*	30 µL
NTC			0 µL	30 µL

3. Load 6.00 µL of master mix to wells on a 96 well plate.
4. Add 4.00 µL ddH_2O to the non-template control (NTC) wells.
5. Transfer 4.00 µL of each concentration in the dilution series to the PCR-plate in triplicate and mix well.
6. Seal the PCR plate with a plastic film and spin down at 2500 rpm.
7. Run the qPCR reaction on a QuantStudio 5 machine (Thermo Fisher Scientific) at the following schedule: 95 °C for 30 sec followed by 40 cycles of: 95 °C for 5 sec, 60 °C for 15 sec and 70 °C for 10 sec. Collect data at end of extension step.

Protocol for RT-qPCR of samples:

- Dilute cDNA to yield a final input of 10 ng (for primers *Nrcam, Phf2* and *Skp1*) or 0,5 ng (for primer *GAPDH*) cDNA into the RT-qPCR reaction.
- Follow steps 3–7 above.

Appendix G - Protocol for Luminex immunoassay

Kit: Rat Stress Hormone Magnetic Bead Panel from Bio-Rad. 96-well Plate Assay. (Cat.no.#RSHMAG-69K).

MILLIPLEX® MAP is based on the Luminex® xMAP® technology.

The method was performed following the manufacturer's instructions.

Preparation of reagents

A. Preparation of Antibody-Immobilized Beads

Add 150 µL from ACTH antibody-bead vial to the Mixing Bottle, in addition to 2.85 mL Assay Buffer.

B. Preparation of Quality Controls

Reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water.

C. Preparation of Wash Buffer

Warm up the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer with 540 mL deionized water.

D. Preparation of Rat Stress Hormone Panel Standard

Reconstituted Standard 7 with 250 µL deionized water.

Prepare Working Standards: Label six polypropylene microfuge tubes Standard 1–6. Add 200 µL of Assay Buffer to each of the six tubes. Add 100 µL of the Standard 7 to the Standard 6 tube, mix well and transfer 100 µL of the Standard 6 to the Standard 5 tube. Repeat this process for the rest of the standards.

Table 18. The concentrations of ACTH in each standard tube after dilutions.

DILUTION	ACTH (PG/ML)
STANDARD 7	1000
STANDARD 6	333.3
STANDARD 5	111.1
STANDARD 4	37.0
STANDARD 3	12.3
STANDARD 2	4.1
STANDARD 1	1.4

Protocol for immunoassay for ACTH:

1. Add 200 µL of Assay Buffer to each well. Seal the plate and mix the contents on a plate shaker for 10 minutes at room temperature (20–25°C).
2. Remove the assay buffer by inverting the plate. Remove residual liquid by tapping the plate onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells.
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of medium without FBS to the background, standard, and control wells.
6. Add 25 µL of Sample into the appropriate wells.
7. Add 25 µL Primary Antibody into all the wells.
8. Vortex the Mixing Bottle and add 25 µL of the Mixed Beads to each well.
9. Seal the plate. Incubate with agitation on a plate shaker overnight (16–18 hours) at 4°C.
10. Remove the contents of the wells, wash the plate 3 times with a wash buffer and a handheld magnet. Method described in the plate washing section.
11. Add 50 µL of Detection Antibodies into each well.
12. Seal the plate and incubate with agitation on a plate shaker for 1 hour at room temperature (20–25°C).
13. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibodies.
14. Seal the plate and incubate with agitation on a plate shaker for 30 minutes at room temperature (20–25°C).
15. Remove the contents of the wells, wash the plate 3 times with a wash buffer and a handheld magnet.
16. Add 100 µL of Sheath Fluid to all the wells. Resuspend the beads on a plate shaker for 5 minutes.
17. Analyze the plate on Bio-Plex® MAGPIX ™ Multiplex Reader, with the software Bio-Plex Manager MP.

Plate washing: Use a Handheld magnet (EMD Millipore Catalog # 40-285). Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, add Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash.

Appendix H - Protocol for SNP genotyping

Reagents:

- DNA (20ng/µL) previously isolated from the saliva samples provided by the subjects in the human cohort.
- Assay/probes rs7209436 (Cat.no. C_1570087_10), rs242924 (Cat.no. C_2257689_10), and rs110402 (Cat.no. C_2544843_10), from Thermo Fisher Scientific.
- TaqMan Genotyping Master Mix (Cat.no. 4371355) from Thermo Fisher Scientific.

Protocol:

1. Thaw the assays/probe.
2. Spin down the 96-well plate with DNA to collect all the content.
3. Swirl the Master mix gently to mix, vortex and centrifuge the assay mix (probe) briefly.
4. Prepare the master mix. Vortex and spin down the mix. Multiply the reagent amount with the number of the samples to prepare the master mix.

Table 19. The components and volume of the master mix for one sample.

REAGENT	1 SAMPLE
TAQMAN GENOTYPING MASTER MIX (2X)	2,50 µL
ASSAYS/PROBE	0,25 µL
TOTAL VOLUME	2,75 µL

5. Add 2.75 µL of the master mix to each well in a 384-well plate by using a multichannel pipette/dispenser pipette.
6. Inspect each well for uniformity, add more master mix to wells with less content.
7. Add 2.25 µL of DNA template according to the prepared loading chart.
8. Seal the plate. Centrifuge the plate for 30 seconds at 2400 rpm.

9. Run the plate according to assay specification on QuantStudio5 (Thermo Fisher Scientific), described below. Use the reporter dyes FAM and VIC to label the probes to distinguish between the two allele variants for each SNP.

QuantStudio5 (from Thermo Fisher Scientific) genotyping cycle:

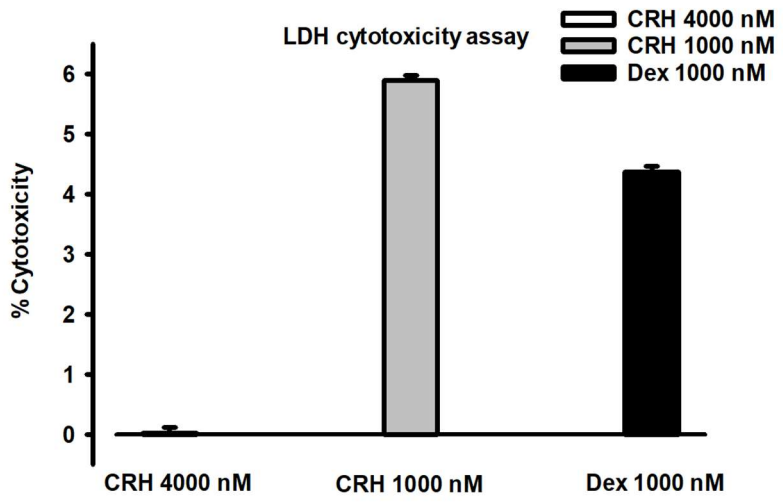
Step 1: 60ºC for 30 minutes.

Step 2: 95ºC for 25 minutes.

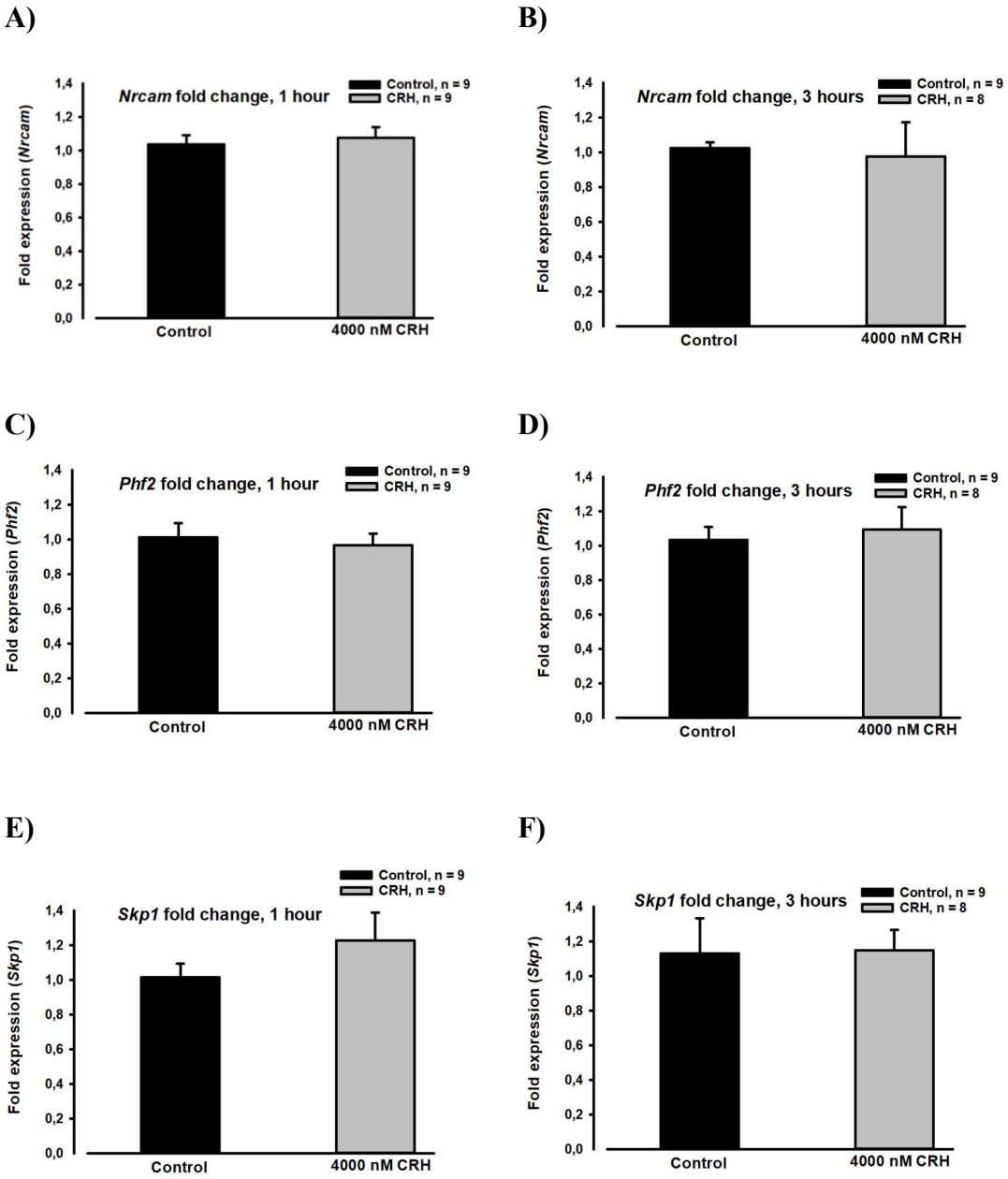
Step 3: 60ºC for 1 hour.

Step 4: 60ºC for 30 minutes.

10. Supplementary



Supplementary figure 1. The LDH cytotoxicity assay. RC-4B/C cells were exposed to two different concentrations of CRH, 1000 nM or 4000 nM, for 3 hours. The cells were exposed to 1000 nM Dex for 3 hours. All data are given as the mean ± SEM.



Supplementary figure 2. The fold expression of the genes Nrcam, Phf2 and Skp1 for cells exposed to 4000 nM CRH: left panel 1 hour, right panel 3 hours. A) and **B)** The Nrcam fold change expression relative to control **C)** and **D)** The Phf2 fold change expression relative to control **E)** and **F)** The Skp1 fold change expression relative to control. All data are given as the mean ± SEM.

Supplementary table 1. A list of the p-values after a statistical analysis with one-way ANOVA on data from samples exposed to 1000 nM CRH or 1000 nM Dex for 1 and 3 hours.

GENE	SAMPLE CONDITION	P-VALUE
NRCAM	1 hour with 1000 nM CRH or 1000 nM Dex	0.966
NRCAM	3 hours with 1000 nM CRH or 1000 nM Dex	0.292
PHF2	1 hour with 1000 nM CRH or 1000 nM Dex	0.759
PHF2	3 hours with 1000 nM CRH or 1000 nM Dex	0.292
SKP1	1 hour with 1000 nM CRH or 1000 nM Dex	0.939
SKP1	3 hours with 1000 nM CRH or 1000 nM Dex	0.621

Supplementary table 2. A list of the p-values after a statistical analysis with the Mann-Whitney rank sum test on data from samples exposed to 4000 nM CRH for 1 and 3 hours.

GENE	SAMPLE CONDITION	P-VALUE
NRCAM	1 hour with 4000 nM CRH	0.724
NRCAM	3 hours with 4000 nM CRH	0.885
PHF2	1 hour with 4000 nM CRH	1.000
PHF2	3 hours with 4000 nM CRH	0.532
SKP1	1 hour with 4000 nM CRH	0.860
SKP1	3 hours with 4000 nM CRH	0.736