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Vliv stresu na regulaci a regeneraci glukokortikoidů u zvířecích modelů lišících se odpovědí osy hypothalamus-hypofýza-nadledviny

The effect of stress on regulation and regeneration of glucocorticoids in animal models differing in response of hypothalamo-pituitary-adrenal axis

Disertační práce

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Prohlášení

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Abstract

Stress reaction is usually activated by the brain, when homeostasis is or perceived to be threatened. The stress signals are transmitted from the brain by two main branches; the sympathoadrenomedullary and the hypothalamo-pituitary-adrenal (HPA) axes and employ neural, humoral and immune pathways to cope with the stressor. Because of its potency, the stress reaction has to be precisely regulated. The HPA axis is regulated by feedback loops where its end product, corticosterone in laboratory rat and mouse, inhibits its activity. The effect of corticosterone does not depend only on the concentration of corticosterone but also on local metabolism of glucocorticoids via oxo-reduction catalyzed by the enzyme 11β -hydroxysteroid dehydrogenase 1 (encoded by the *Hsd11b1* gene), which intracellularly regenerates active corticosterone from inactive 11-dehydrocorticosterone, or by extra-adrenal de novo steroidogenesis of glucocorticoids. We focused on analysis of stress response in experimental animals differing in HPA axis responsivity (Fischer 344 rats (F344) vs. Lewis rats (LEW) and germ-free (GF) vs. specific pathogen free mice (SPF)) with special emphasis on regulation of stress response, glucocorticoid regeneration and influence of gut microbiota. We found that stress modulated local regeneration of glucocorticoids in the limbic structures involved in HPA axis regulation but not in the canonical structures of HPA axis. F344 and LEW rats showed differences in stress-dependent changes of expression of genes involved in HPA axis regulation in limbic areas. Similarly, psychosocial stress upregulated regeneration of corticosterone in lymphoid organs and this effect was more pronounced in LEW than F344 rats. Similarly, inflammatory stress elevated glucocorticoid regeneration in specific microanatomical compartments of the murine gut immune system and expression of *11hsdb1* correlated with the expression of *Tnfα* as well as other cytokines. Microbiota modulated behavior in social conflicts and the response of the HPA axis, colon and mesentery lymph nodes to chronic psychosocial stress. We also demonstrated that microbiota impact the response of the pituitary, adrenals and intestine to acute restraint stress. Together we can conclude that local regeneration of glucocorticoids plays an important role in central feedback regulation of HPA axis response and in local restriction of immune system. The microbiota are involved in modulation not only the HPA response to stress but also behavior and local extra-adrenal glucocorticoid regeneration and de-novo synthesis.

Abstrakt

Mozek aktivuje stresovou odpověď v situacích, kdy je nebo se zdá být ohrožena homeostáza. Informace o stresu jsou vedeny z mozku dvěma hlavními větvemi; sympatoadrenálním systémem a osou hypothalamus-hypofýza-nadledviny (HPA), které aktivují neurální, humorální a imunitní dráhy, určené pro zvládání stresových situací. Protože se jedná o velmi účinný mechanismus, musí být stresová odpověď přesně řízena. HPA osa je regulována zpětnovazebným systémem, kdy její konečný produkt, kortikosteron u laboratorních potkanů a myší, tlumí její aktivitu. Efekt kortikosteronu nezávisí pouze na jeho koncentraci, ale také na lokálním metabolismu glukokortikoidů katalyzovaném enzymem 11β-hydroxysteroiddehydrogenázou 1 (kódovanou genem *Hsd11b1*), který obnovuje kortikosteron z 11-dehydrokortikosteronu uvnitř buňky; nebo *de novo* syntézou glukokortikoidů. V naší práci jsme se zaměřili na zkoumání stresové odpovědi u pokusných zvířat lišících se reaktivitou HPA osy ((potkani kmene Fischer 344 (F344) proti potkanům kmene Lewis (LEW) a bezmikrobní (GF) myši proti myším bez specifického patogenu (SPF)), se zaměřením na regulaci stresové odpovědi, regeneraci glukokortikoidů a vliv mikrobioty. Zjistili jsme, že stres moduluje lokální regeneraci glukokortikoidů v limbických oblastech zapojených do řízení HPA osy, ale nemá vliv v jednotlivých složkách samotné HPA osy. Kmeny potkanů F344 a LEW vykazovaly různé stresem indukované změny genů podílejících se na regulaci HPA osy v limbických oblastech. Obdobně, stres zvýšil regeneraci glukokortikoidů v lymfatických orgánech a toto zvýšení bylo více zřetelné u kmene LEW než u kmene F344. Regenerace glukokortikoidů byla také zvýšena zánětem ve specifických mikroanatomických kompartmentech myšího střevního imunitního systému a exprese *Hsd11b1* korelovala s expresí *Tnfα* a některých dalších cytokinů. Mikrobiota modulovala chování v sociálním konfliktu a odpověď HPA osy, tlustého střeva a mezenteriálních lymfatických uzlin při vystavení chronickému psychosociálnímu stresu. Mikrobiota rovněž ovlivňovala odpověď hypofýzy, nadledvin a střev na akutní stres znehybněním. Celkově lze uzavřít, že lokální regenerace glukokortikoidů hraje důležitou roli v centrální zpětnovazebné regulaci odpovědi HPA osy na stres a v lokální regulaci imunitního systému. Mikrobiota se účastní nejenom na modulaci odpovědi HPA osy při stresu, ale také chování a lokální extra-adrenální regenerace glukokortikoidů a jejich syntézy *de novo*.

List of abbreviations

The proteins are written in capital letters, genes are written in italics with the first capital letter followed by small letters. Abbreviation of genes are stated only, when the name of the gene differs from name of the protein.

11HSD1	11β-hydroxysteroid dehydrogenase type 1
11HSD2	11β-hydroxysteroid dehydrogenase type 2
3βHSD	3β-hydroxysteroid dehydrogenase
5-HT	seretonin
ACTH	adrenocorticotropic hormone
AG	adrenal glands
BBB	blood-brain barrier
BLA	basolateral amygdala
BNST	bed nucleus of stria terminalis
CA2	cornu Ammonis 2 subfield of the hippocampus
CA3	cornu Ammonis 3 subfield of the hippocampus
CCE	colonic crypt epithelium
CeA	central amygdala
CRH	corticotropin-releasing hormone
CRHR1	corticotropin-releasing hormone receptor 1
CRHR2	corticotropin-releasing hormone receptor 2
CVOs	circumventricular organs
CYP11A1	cholesterol side-chain cleavage enzyme (P450scc)
CYP11B1	11β-hydroxylase (P450c11b1)
CYP17	17α-hydroxylase (or P450 17A1)
CYP21	steroid 21-hydroxylase (P450c21)
Dax-1	dosage-sensitive sex reversal, adrenal hypoplasia critical region
DBD	DNA-binding domain
dCA1	dorsal portion of cornu Ammonis 1 subfield of the hippocampus
ENS	enteric nervous system
F344	Fischer 344 rat strain
FKBP5	FK506 binding protein 51
FST	forced swim test
GF	germ free
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA	hypothalamo-pituitary-adrenal
Hsd11b1	gene encoding 11β-hydroxysteroid dehydrogenase type 1
Hsd11b2	gene encoding 11β-hydroxysteroid dehydrogenase type 2

IL	interleukin
ILF	isolated lymphoid follicles
ilPFC	infralimbic prefrontal cortex
LA	lateral amygdala
LBD	ligand-binding domain
LEW	Lewis rat strain
LPS	lipopolysacharides
LRH-1	liver receptor homolog-1
LTP	long-term potentiation
MC2R	melanocortin 2 receptor (Receptor for ACTH)
ME	median eminence
MLN	mesenteric lymph node
mPFC	medial prefrontal cortex
MR	mineralocorticoid receptor
nGRE	negative Glucocorticoid response element
Nr3c1	gene encoding the glucocorticoid receptor
NTD	amino-terminal domain
NTS	nucleus of the solitary tract (nucleus tractus solitarii)
OXT	oxytocin
P450 17A1	17α-hydroxylase (or CYP17)
P450c11b1	11β-hydroxylase (CYP11B)
P450c21	steroid 21-hydroxylase (CYP21)
P450scc	cholesterol side-chain cleavage enzyme (CYP11A1)
PAC1	receptor for PACAP
PACAP	pituitary adenylate cyclase activating polypeptide
PFC	prefrontal cortex
plPFC	prelimbic prefrontal cortex
POMC	pro-opio-melanocortin
PVN	paraventricular nucleus of hypothalamus
PVT	paraventricular nucleus of the thalamus
SAM	sympathoadrenomedullary
SDRs	short-Chain Dehydrogenases/Reductases
SF-1	steroidogenic factor 1
SPF	specific pathogen free
StAR	steroidogenic Acute Regulatory Protein
TNF	tumor necrosis factor
TRFs	transcriptional regulatory factors
UCN	urocortin
V1B	vasopressin receptor type V1B also called V3
vCA1	ventral portion of cornu Ammonis 1 subfield of the hippocampus

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1 Introduction

1.1 General introduction

To successfully survive and procreate in unpredictable world, animals (as well as humans) have to cope with various challenges including predators, shortage of nutrients, intra-species rivals, bad weather, infections etc. To do so, the organisms evolved mechanisms to maintain homeostasis in dynamic environment, the stress response. The founder of stress as a scientific concept, Hans Selye defined stress response as the nonspecific response of the body to any demand (Selye 1950). However, later it was shown, that different stressors evoke distinct central neurochemical and peripheral neuroendocrine patterns of response (Pacak et al., 1998). Recently, the stress response was defined as a reaction of the organism to stimulus (stressor) that threatens homeostasis (or is perceived as a threat to the homeostasis by the organism) and is aimed to regain homeostasis (Chrousos 2009; Pacak and Palkovits 2001). Stress is often regarded as something pathological however, it is important to realize that acute stress response represents a physiologically important emergency tool, which is designed to apply powerful means when the organism's health or existence are at stake. The ability of the organism to actively maintain homeostasis in changing environment is called the "allostasis". The adverse effects of stress come to play, when stressors remain for longer periods of time and/or individual management of the stress response is impaired. The cost of wear and tear of the stress reaction is referred to as allostatic load (McEwen and Gianaros, 2011).

The stress response originates in the brain and employs all systems needed to fight for survival, including cardiovascular, metabolic and immune systems. The stress signals are transmitted from the brain by two main branches; the sympathoadrenomedullary (SAM) axis and the hypothalamo-pituitary-adrenal (HPA) axis. The response of the SAM axis is employed within seconds after stressor insult and comprises elevated heart rate, increased blood pressure, increased glycaemia etc. and is accompanied by the elevation of catecholamines. The HPA axis response is slower, however its effectors (corticosterone in rodents, cortisol in humans) have longer plasma half-life. Both axes are aimed to maintain and restore homeostasis and cover energy expenditure needed to escape from dangerous situation (Ulrich-Lai and Herman, 2009).

The stress response is primarily aimed on survival, thus applying such powerful mechanism is not without consequences. The founder of the term "stress", Hans Selye, has described that

severe and long lasting stressors result in adrenal hypertrophy, atrophy of thymus (and lymph nodes) and erosions of gastrointestinal tract (gastroduodenal ulcers) (Selye, 1950; Szabo et al., 2017). Since that time, extensive research of the effects of stressors on organisms has revealed, that over-activation of stress response can contribute to the development of neuropsychiatric and cardiovascular disorders and disturbances of immune system (Bellavance and Rivest 2014; Dhabhar, 2014; Elenkov and Chrousos, 2002; McEwen, 2006; Steptoe and Kivimaki, 2012).

The stress response is governed by brain and also greatly affects the brain itself in several aspects. The brain evaluates the danger using external and internal signals, as well as anticipatory response. In return, the stress related signals influence brain and can alter memory formation and store contextual memories to avoid dangerous situations in future (Roozendaal et al., 2009). It is known from experience, that small to medium levels of stress could be sometimes beneficial to promote learning, growth and adaptation. In older literature it was referred to as "Eustress" and the damaging stress was called "Distress" (Selye, 1975). The effect of stressors on performance is usually displayed as inverted U-shaped curve, sometimes called the Yerkes-Dodson Law. The performance increases with higher stress, motivation or anxiety to a certain point, from which further increasing of stress, motivation or anxiety interfere with cognitive processes and leads to decreased performance (Chrousos, 2009). However, this paradigm is valid only for more complex tasks. For easy tasks, the curve reaches plateau and does not fall (Figure 1) (Diamond et al., 2007).

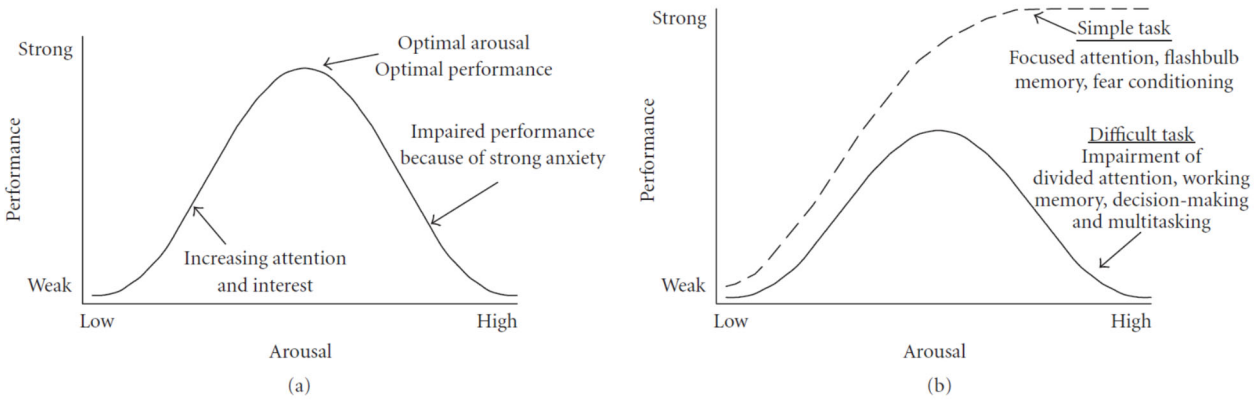

Figure 1. Relationship between arousal and cognitive performance. Generally accepted view (a) of U-shaped curve (Chrousos, 2009) that usually neglects relationship between stress and performance for easy tasks and original (and still valid) observation by Yerkes and Dodson (1908) (b), that in simple tasks high emotionality can enhance performance and that interference between high emotionality and performance is valid only for more complex task. From Diamond et al., 2007.

It is proposed that in stressful situation, the brain shifts from higher order (hippocampal) to habitual (striatum based) responses (Goldfarb and Phelps, 2017), and thus easy tasks are still

manageable. Regarding harmful effects of stress, recent findings emphasized the role of perceived controllability and predictability of the stressor in adaptation and perceived severity of the stressor (Koolhaas et al. 2011). It is also is known, that the same stressor may not have the same effect on all individuals. This individual variability in susceptibility to stressors is probably due to the tradeoff between traits. The unpredictability of environment and evolutionary pressure maintains variability between traits. The relative advantage of a trait usually depends on many variables, such as food availability, population density or predators and climatic disturbances. Different conditions requires different traits in order to successfully cope with these conditions, and it is not possible to say, that one trait is universally ideal. For example, an individual that is hyper-reactive to stress, will have advantage in its readiness to escape dangerous environment, but at the cost of allostatic load and possible exhaustion. On the other hand, hypo-reactive animals could be endangered by autoimmune or inflammatory diseases (Bellavance and Rivest, 2014; Korte et al., 2005; Sternberg et al., 1989, 1992). The genetically determined predisposition to stress reactivity can also be modulated by many external factors such as previous experiences of stress exposure, sleep, diet, or microbiome composition. Microbiome was identified as one of the factors shaping both endocrine and behavioral responses to stressors (Cryan and Dinan 2012; Foster et al. 2017). Therefore it is important to study the stress response and dissect the mechanisms of its action in order to improve therapy and prevention of stress related diseases.

1.1.1 **Overview of the HPA axis**

The hypothalamo-pituitary-adrenal (HPA) axis is the principal endocrine component of stress response and a self-regulatory pathway that utilizes its end-products (cortisol and corticosterone) to control its own activation and responsiveness through a negative feedback mechanism (Figure 2). The initiation of HPA axis activation is controlled by the parvocellular neurons located in the paraventricular nucleus (PVN) of the hypothalamus. However, PVN is influenced by central stress excitatory and inhibitory circuits that integrate stress-related signals from both intrahypothalamic and extrahypothalamic structures (Ulrich-Lai and Herman, 2009). When activated, the release of corticotrophin-releasing hormone (CRH) in the median eminence (ME) is employed and production of CRH is initiated in the medial parvocellular neurons of the PVN (Aguilera and Liu, 2012).

CRH released from ME is transported via portal blood stream to the anterior pituitary, where it binds to the corresponding receptor (CRHR1) activating adenylate cyclase, which leads to the release of adrenocorticotropic hormone (ACTH). Binding of CRH also increases the expression of the pro-opio-melanocortin (POMC) gene. Its gene product is a large preprohormone, which is subsequently cleaved by prohormone convertase 1 in several peptides: resulting in the production of N-terminal peptide, joining peptide, ACTH, β-endorphin, and β-lipotropin. Action of CRH in the anterior pituitary is enhanced by arginine-vasopressin, which is co-released with CRH in ME and activates via arginine-vasopressin 1B receptors (V_{1B}) the protein kinase C in anterior pituitary. Activation of POMC transcription and subsequent release of ACTH are contingent on the type of stressor that causes the initial stress response (Herman et al. 2016).

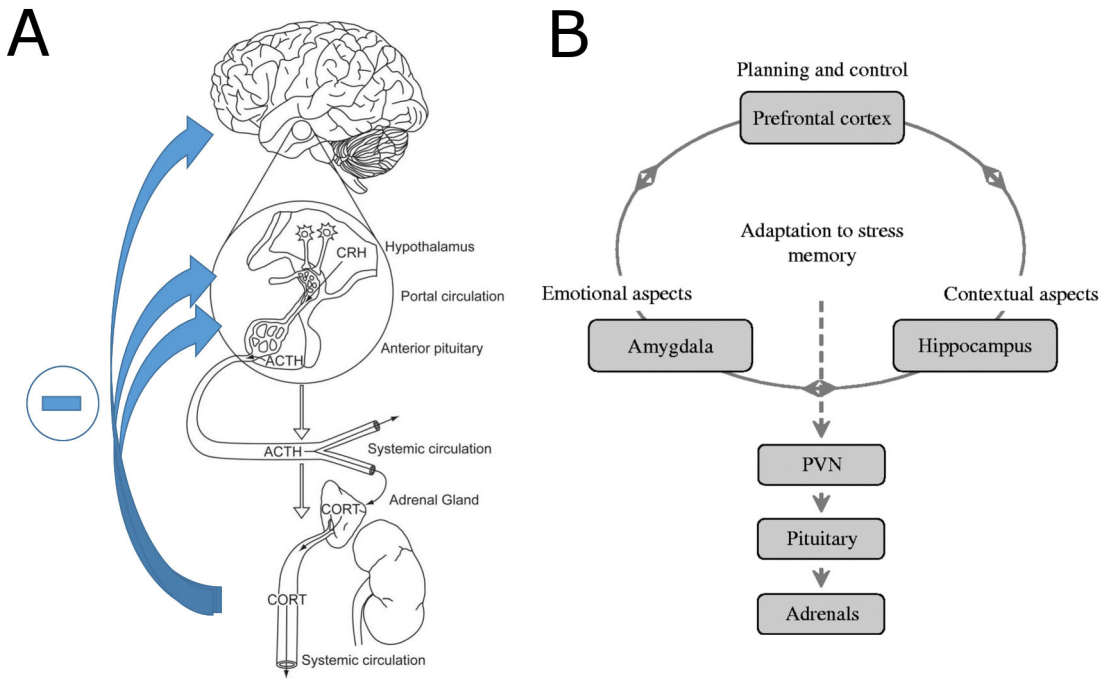

Figure 2: Regulation of the HPA axis. (A) The HPA axis is inhibited by its end-products glucocorticoids at several levels of HPA axis including pituitary and PVN (adapted from Herman et al., 2016). (B) Control of the HPA axis by the limbic system. The hormones of the HPA-axis coordinate information processing and promote connectivity between amygdala, prefrontal cortex and hippocampus to facilitate behavioural adaptation. Projections from the limbic structures innervate the PVN network and regulate trans-synaptically the activity of the HPA-axis (Groeneweg et al., 2011).

ACTH released to the blood stream is transported to adrenal glands (AG), where it activates melanocortin 2 receptors (MC2R). In contrast to other melanocortin receptors, MC2R is activated exclusively by ACTH and is expressed predominantly in zona fasciculata and reticularis of adrenal glands (Gantz and Fong 2003). Binding of ACTH to MC2R results in activation of cAMP-protein kinase A signaling pathway, which leads to acute increase of expression and function of the

steroidogenic acute regulatory protein (StAR), a protein which facilitates movement of cholesterol (precursor of glucocorticoids) from outer to inner mitochondrial membrane (Clark, 2016; Miller and Auchus 2011). As StAR is produced de novo after trophic hormone stimulation of the target cells, the StAR-mediated transport of cholesterol represents one of the rate limiting steps of glucocorticoid production (Clark et al. 1994). The cytochrome P450scc (cholesterol side chain cleavage enzyme) located in the mitochondrial matrix, catalyzes the conversion of cholesterol to pregnenolone and represents another rate-limiting step in steroidogenesis. P450scc is encoded by the *CYP11A1* gene whose expression is hormonally regulated (Miller and Auchus, 2011). Pregnenolone is further converted into progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD). Another enzyme from cytochrome P450 family, steroid 21-hydroxylase (P450c21; *CYP21*) catalyzes hydroxylation of progesterone to 11-deoxycorticosterone. The last step in rats and mice is β-hydroxylation of 11C leading to corticosterone, which is catalyzed by 11β-hydroxylase (P450c11b1; *CYP11B1*) (Payne and Hales 2004). In human adrenals, the enzyme 17α-hydroxylase (P450 17A1; *CYP17*) is present and thus pregnenolone is converted to 17α-hydroxypregnenolone and progesteron to 17α-hydroxyprogesterone, which is further converted by CYP21 and CYP11B1 to cortisol (Payne and Hales, 2004).

1.1.1.1 Metabolism of glucocorticoids

In plasma, corticosterone or cortisol are bound to transcortin (cortisol-binding globulin, CBG) and to lesser extent to albumin. Only about 5 – 10 % of cortisol is free and available for physiological activity and metabolic degradation. The plasma half-life of cortisol in humans is 60 – 90 min (Hall et al. (2010). In contrast, the plasma half-life of total corticosterone in rats is 25 min (Sainio et al. 1988). The major site of glucocorticoid degradation is the liver, although other tissues of the body are also capable of cortisol catabolism. The end-products of degradation are conjugated with glucuronic acid. About 25 % of degraded glucocorticoids are excreted into the bile and then feces, the remaining conjugates formed by the liver enter the circulation and as highly soluble substances they are filtered readily in the kidneys and excreted into the urine.

1.1.1.2 Receptors for glucocorticoids

The glucocorticoids, corticosterone in rodents and cortisol in humans, acts on glucocorticoid receptor (GR). Together with mineralocorticoid receptor (MR), progesterone

receptor, estrogen receptor and androgen receptor, GR belong to the superfamily of nuclear receptors activated by ligands, which operate as transcription factors (Heitzer et al., 2007). The GRs are expressed ubiquitously throughout the body and orchestrate intracellular responses leading to the changes in metabolism, immune system, vascular tone and central nervous system (Revollo and Cidlowski 2009). It is estimated that there are between 1,000 and 2,000 genes that are subject to GR-mediated regulation, with some studies stating that up to 20 % of all genes are GR-responsive (Galon et al., 2002; Weikum et al. 2017). The importance of these receptors for survival has been shown by deletion of GRs, which leads to the developmental abnormalities and death shortly after birth (Cole et al. 1995). The GR is a modular protein, which comprised the amino-terminal domain (NTD), the DNA-binding domain (DBD), and the C-terminal ligand-binding domain (LBD). The protein is encoded by *Nr3c1* gene and can be subjected to splicing and post-translational modification including phosphorylation, sumoylation, ubiquitylation, acetylation and nitrosylation (Figure 3) (Timmermans et al., 2019; Weikum et al., 2017).

The mature GRs are found in cytoplasm in a monomeric state bound to complexes of accessory proteins and the whole complex participates in translocation of activated GR to the nucleus (Timmermans et al., 2019). The ligand-activated receptor binds to the glucocorticoid response element (GRE), usually in the form of dimers, to induce transactivation (GRE) or transrepression (nGRE). GR can also regulate gene activity independent of DNA binding via protein–protein interactions with other transcription factors (Scheschowitsch et al., 2017).

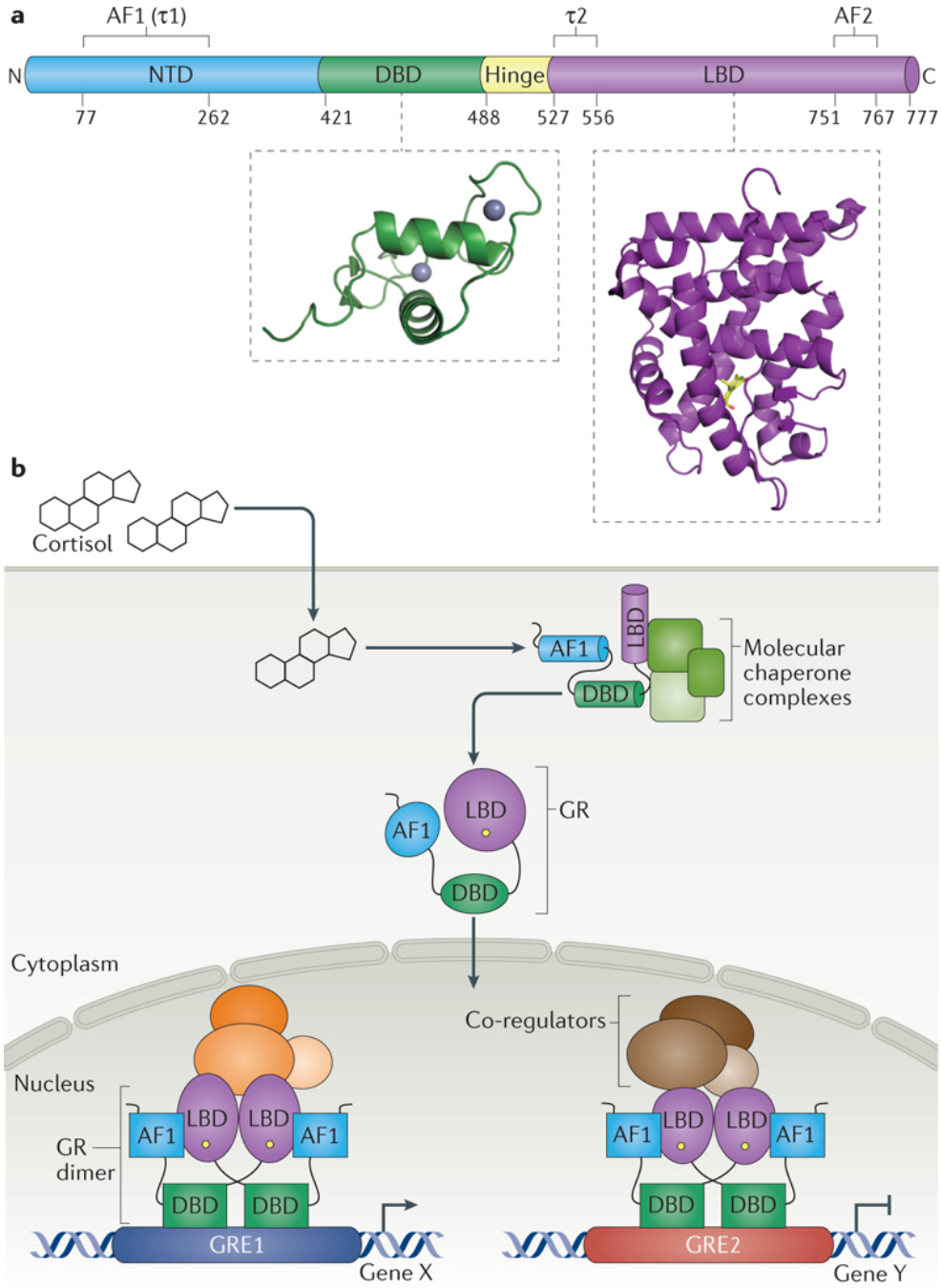

Figure 3. GR singnalling and DNA binding. Linear domain structure of glucocorticoid receptor (GR). (a) GR comprises of the amino-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand-binding domain (LBD). (b) Overview of signaling mediated by natural GR ligand cortisol. Activating ligand interacts with monomeric GR associated with molecular chaperone-containing complexes in the cytosol. This induces local and remote allosteric changes that potentiate nuclear transport and other activities. Within the nucleus, GR nucleates multi-component transcription regulatory complexes containing various other transcriptional regulatory factors (TRFs) and transcriptional co-regulators at different glucocorticoid response elements (GREs) to activate or repress transcription of particular target genes. GRE1 and GRE2 represent distinct GREs within the genome, Gene X and Gene Y represent the genes under the control of GRE1 and GRE2, respectively. From Weikum et al., 2017

Some glucocorticoid-induced responses are too fast to be associated with genomic effect. Membrane-bound glucocorticoid receptors that promote non-genomic actions of glucocorticoid and their function are currently discussed (Deng et al., 2015; Groeneweg et al., 2011; Strehl and Buttgereit, 2014; Tasker et al., 2006).

The glucocorticoids also activate the MRs. These receptors have higher affinity to glucocorticoids than GR and thus the activation of MR by glucocorticoids is protected in mineralocorticoid target tissues by intracrinic modulation of glucocorticoid signals by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11HSD2) (see chapter 1.1.2.1).

In brain, the GRs are expressed ubiquitously, whereas MRs are expressed predominantly in limbic areas (amygdala, hippocampus). MRs have ten times higher affinity for glucocorticoids than GRs (Reul and deKloet 1985) and expression of 11HSD2 is generally low in the brain. Thus, the MRs are predominantly occupied by glucocorticoids at basal conditions, whereas GRs respond to circadian and stress-induced peaks of corticosterone (deKloet et al., 2005; Mifsud and Reul, 2018). Both GRs and MRs participate in HPA axis regulation and stress-induced memory and behavior (McEwen, 2007). The importance of intact glucocorticoid signalling in brain was demostrated by the deletion of GR in the forebrain (regions encompassing the cerebral cortex, hippocampus, nucleus accumbens, caudate–putamen, basolateral and basomedial amygdala, and bed nucleus of the stria terminalis) which was accompanied by number of physiological and behavioral abnormalities that mimic depressive disorders (Boyle et al., 2006).

1.1.1.3 Effects of glucocorticoids

As their name suggests, glucocorticoids are known for their effect on carbohydrate metabolism. Glucocorticoids stimulate hepatic gluconeogenesis, increase mobilization of amino acids from other tissues and mobilization of fatty acids. This leads to mild increase of glycaemia and subsequent stimulation of insulin secretion, which, if prolonged, can lead to insulin resistance. Moreover, glucocorticoids have permissive effect for other hormones such as catecholamines and glucagon. Metabolic effects are important not only after stressful challenges but also in basal state; the diurnal fluctuation of glucocorticoids controlled by circadian clock and prepares organism for regular peaks of activity (Dickmeis, 2009). Glucocorticoids are also known for their immunomodulatory and immunosuppressive properties. High levels of glucocorticoids are used in clinical practice for their anti-inflammatory actions. Glucocorticoids modulate production of pro-

inflammatory cytokines (including IL-1B, TNFa, IL-6, IL-8, IL-12, and IL-18 etc.) and modulators (eg. COX-2, iNOS). They also increase expression of other transcriptional regulators, such as glucocorticoid-induced leucine zipper, which regulates immune response at several levels, including inhibition of translocation of pro-inflammatory factor NF-kB, restraining skin inflammation mediated by IL-17 and participation in apoptosis of neutrophils (Petrillo 2017). Glucocorticoids are shifting immune response towards humoral (Th2) immunity by participating in maturation and function of IL-10 producing T-cells and directly enhancing IL-10 secretion by macrophages and dendritic cells (Franchimont 2004).

1.1.2 **Regulation of the HPA axis**

There is a negative feedback regulation mediated by glucocorticoids at all levels of the HPA axis (Figure 2). In the PVN, corticosterone inhibits both synthesis and secretion of CRH (Aguilera et al., 2007, Harbuz and Lightman 1989). Increased expression and secretion CRH in PVN and exaggerated CRH response to minor stressor was described in adrenalectomized rats (Ma and Aguilera 1999). The exact mechanism of glucocorticoid action on CRH neurons is not clear. Negative GRE was found *in vitro* using AtT-20 cells transfected with the human CRH gene (Malkoski and Dorin, 1999) but the feedback mechanism is at least partially maintained also by membrane glucocorticoid receptors of CRH neurons in PVN that mobilize the synthesis of endocannabinoids. Endocannabinoid release then causes presynaptic inhibition of glutamate release, which reduces the neural activity of parvocellular neurons (Di et al., 2003, Tasker et al., 2006).

In pituitary, the glucocorticoids activate their receptors, which bind to the nGRE on the promoter of POMC gene and, thus inhibits POMC transcription. Moreover, glucocorticoids promote translocation of Annexin1, which inhibits CRH-induced ACTH secretion. In addition, several other mechanisms of glucocorticoids inhibition of ACTH synthesis and secretion were proposed (Deng et al., 2015; Gjerstad et al., 2018).

1.1.2.1 Intracellular modulation of glucocorticoid activity

The response of target tissue depends not only on the concentration of glucocorticoids and density of receptors, but also on the pathways how glucocorticoid signal can be modified inside the cells. The FK506 binding protein 51 (FKBP5) is a co-chaperone of HSP 90 and belongs to immunophilin family. When bound to the glucocorticoid receptor complex, it decreases its affinity

to glucocorticoids and GR translocation to the nucleus. FKBP5 is part of ultra-short negative feedback loop, where activation of GR increases expression of FKBP5 decreasing thus GR activity. Overexpression of FKBP5 and subsequent decreased glucocorticoid feedback is associated with depressive behavior (Binder 2009, Gjerstad et al., 2018).

The amount of glucocorticoids available for GR or MR can be influenced by enzyme 11β-hydroxysteroid dehydrogenase (11HSD). This enzyme belongs to the Short-Chain Dehydrogenases/Reductases (SDRs) superfamily, catalyzing NAD(P)(H)-dependent oxidation/reduction reactions (Figure 4). The coenzyme binding is located to the N-terminal part, while the substrate binding is located to the C-terminal part (Persson et al., 2003). Two isoform of this enzyme have been characterized: 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) and type 2 (11HSD2). It was shown that 11HSD1 has both dehydrogenase and reductase activity however, *in* vivo when the cells are not disrupted, it has predominantly reductase activity (Tomlinson et al., 2004). The 11HSD1 is located in the endoplasmic reticulum and amplifies intracellular glucocorticoid action by converting biologically inactive 11-oxo-steroids (cortisone, 11-dehydrocorticosterone) to biologically active cortisol and corticosterone (Tomlinson et al., 2004; Wyrwoll et al., 2011). This enzyme is expressed in the brain (Holmes and Seckl, (2005); Wyrwoll et al., 2011), pituitary gland (Hanafusa et al., 2002), adrenal gland (Shimojo et al., 1996) and many other peripheral organs (Tomlinson et al., 2004). The potential of 11HSD1 in regulation of glucocorticoid signal in brain was demonstrated by application of 11HSD1 inhibitor, which prevented stress-induced suppression of hippocampal synaptic potentiation and impaired contextual, but not tone-cue fear conditioning (Sarabdjitsingh et al., 2014). We found that *Hsd11b1* expressoin is up-regulated by stress in limbic areas of brain and participates in the control of HPA axis activity, but not in the HPA axis itself (Ergang et al., 2015; Vodička et al., 2014).

On the other hand, 11HSD2 catalyzes the oxidation of cortisol and corticosterone to inactive cortisone and 11-dehydrocorticosterone (Figure 5) and reducing the local glucocorticoid signals (Wyrwoll et al., 2011). It is expressed predominantly in kidney, placenta, salivary and sweat glands. These tissues are target for mineralocorticoids or could be potentially harmed by glucocorticoid excess (Tomlinson and Stewart, 2001). 11HSD2 was also found in moderate amounts in brain loci involved in regulation of sodium appetite and blood pressure regulation, such as NTS and some hypothalamic nuclei (Wyrwoll et al. 2011).

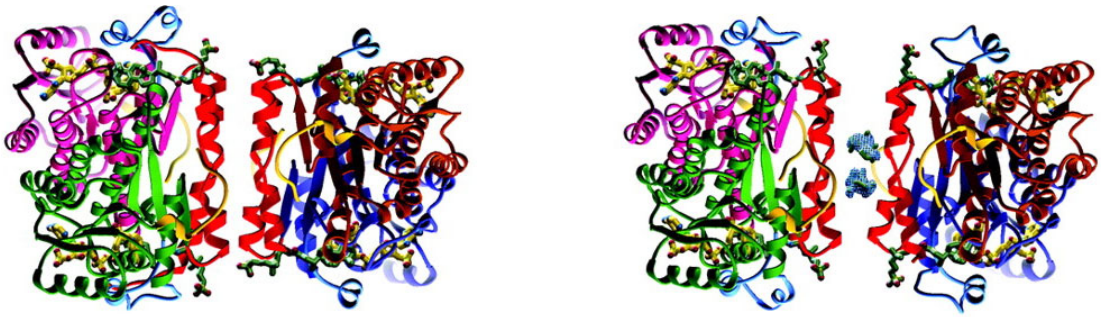

Figure 4. Quaternary association of 11β hydroxysteroid dehydrogenase type 1 (11HSD1) subunits and associated interactions and conformational changes. Overall topology of the 11HSD1 interface-closed (left) and interface-open (right) tetramers. From Hosfield et al. 2005

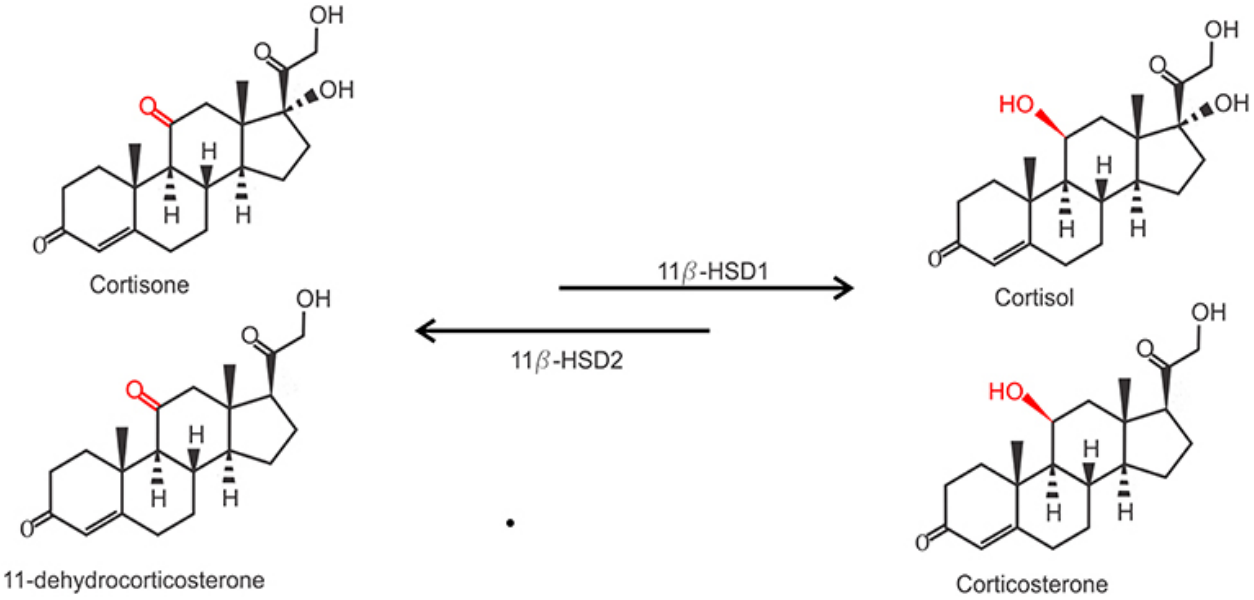

Figure 5. Conversion of inactive GCs to active GCs. Inactive cortisone (human) and 11-dehydrocorticosterone (mouse) are activated to active cortisol and corticosterone by 11β hydroxysteroid dehydrogenase type 1 (11HSD1), and inactivated again by 11HSD2. Timmermans et al. 2019.

1.1.3 **Central regulation of HPA axis**

As the primary controller of HPA axis, the PVN integrates variety of information from external and internal sources. The information coming to the PVN can be divided to systemic and psychogenic responses. The systemic response represents sensory signals from the body (including somatic and visceral pain), neural homeostatic signals (chemoreceptors, baroreceptor, osmoreceptors), humoral signals (glucose, leptin, insulin, renin-angiotensin etc.), humoral inflammatory signals (IL-1, IL-6, TNF-a and others), whereas the psychogenic responses originate from higher order cognitive areas and are based on innate responses (predators, unfamiliar

environments, social challenges) and memory-generated (conditioned) triggers (Herman et al., 2003). Both systemic and psychogenic stimuli are processed in multiple limbic areas, including prefrontal cortex, amygdala and hippocampus (Figure 2). In general, the limbic system is involved in emotional and motivational processing, learning, memory and coordination of behavioral responses to stress and participates in the HPA axis regulation (Herman 2013, Morgane et al., 2005). Although missing direct projections to PVN, the output of these limbic structures converges on crucial subcortical relay sites; most notably nucleus tractus solitarii and bed nucleus of stria terminalis; which allow further downstream processing of limbic information (Ulrich-Lai and Herman, 2009).

1.1.3.1 Medial prefrontal cortex

The medial prefrontal cortex (mPFC), interconnected with the hippocampus and the amygdala, plays an important role in coordination of behavioral and physiological stress responses across multiple temporal and contextual domains (McKlveen et al., 2015; Ulrich-Lai and Herman 2009). Acute stressors activate c-fos expression in mPFC (Cullinan et al. 1995; Morrow et al., 2000; Ostrander et al., 2003) while chronic stress as well as high glucocorticoids lead to changes in dendritic architecture of the mPFC (Cook and Wellman, 2004; Radley et al., 2004, 2005 Wellman et al., 2001). Intact mPFC is important for negative HPA axis feedback and corticosterone implants to mPFC regions decrease corticosterone levels (Akana et al., 2001; Diorio et al., 1993). In addition, specific roles of dorsal and ventral sub-regions of mPFC were shown in the regulation of HPA axis during stress (Radley et al. 2006). It seems that glucocorticoids play important role in this process, as knockdown of GR in prelimbic prefrontal cortex (plPFC) led to hyperresponsivity to acute stress, whereas GR knockdown in infralimbic prefrontal cortex (ilPFC) resulted in hyper-responsiveness both to acute and chronic stressors (McKlveen et al. 2013). The inhibitory role of ilPFC in regulation of the HPA axis in both acute and chronic stress is dependent on glutamate output (Myers et al., 2017).

1.1.3.2 Amygdala

Amygdala is a complex of nuclei, which are best known for involvement in fear responses and memory consolidation and are tightly related to stress. Moreover, it is also considered as a key node for stress integration thanks to its involvement in autonomic regulation. Amygdala is a complex structure with numerous downstream targets that modulate autonomic and

neuroendocrine stress responses (Davis, 1992; Roozendaal et al. 2009, Ulrich-Lai 2009). Amygdala is also considered as one of the brain areas involved in stressor/modality specific response (Dayas et al., 2001; Figueiredo et al., 2003; Prewitt and Herman, 1997). In contrast to PVN, where glucocorticoid exerts negative feedback, the glucocorticoids increase the activity of amygdalar CRH system (Kovacs 2013; Makino et al., 1994; Zalachoras et al., 2016). The effect of amygdala on PVN is mediated mostly via the bed nucleus of the stria terminalis (BNST) (Choi et al. 2007).

1.1.3.3 Hippocampus

Based on anatomical, molecular and behavioral data, the hippocampus can be divided into two functionally distinct parts. The dorsal portion performs mostly cognitive tasks, for example spatial navigation and memory, whereas the ventral part is related to emotions, stress and affect (Fanselow and Dong, 2010). The hippocampus is also important for inhibition of the HPA axis. GRs and MRs are involved in this process. MRs exert tonic inhibitory influence on the activity of the PVN neurons in the hypothalamus and GR are responsible for negative feedback action of glucocorticoid hormones (de Kloet et al., 2005; Reul et al., 2015). The effect of hippocampus on HPA axis inhibition is most pronounced during the recovery phase of stress-induced glucocorticoid secretion, implicating the hippocampus in the regulation of termination of stress-initiated HPA responses. The signals from hippocampus to PVN are driven trans-synaptically and preferentially through distinct populations of GABA-ergic neurons in the BNST (Herman 2003; Ulrich-Lai and Herman 2009).

1.1.3.4 Neuromodulation of the HPA axis

Distinct neuronal circuits can be influenced by various neurotransmitters, neuromodulators and stress mediators, which are released during stress. As summarized by Joëls and Baram (2009), numerous neuropeptides are released by stress in specific populations of neuronal cells and contribute to the activation of the stress response or counteract it. CRH, the principal peptide in HPA axis activation, is expressed in the PVN of the hypothalamus. Besides the hypothalamus, CRH is widely distributed in extrahypothalamic circuits of the brain where it, together with other peptides of "the CRH family" (urocortins UCN1, UCN2 and UCN3), functions as a neuromodulator establishing and integrating a complex humoral and behavioral system that regulates multiple aspects of the stress response (Inda et al. 2017). Receptors for CRH, (CRHR1

and CRHR2) are expressed in PVN and amygdala and they play important role in the regulation of stress response (Jamieson et al., 2006; Tanaka et al., 2003). CRHR1 binds CRH and UCN1 with higher affinity, whereas CRHR2 preferably binds UCN2 and UCN3 (Bale and Vale, 2004). Another peptide that is released by stress and is involved in the HPA axis regulation is oxytocin (OXT), which reduces physiological and behavioral indices of stress (Engelmann et al. 2004; Lee et al., 2009; Winter and Jurek, 2019). The pituitary adenylate cyclase-activating polypeptide (PACAP), is a pleiotropic neuropeptide that represents an important regulator of neuroendocrine stress response pathways in the brain (Lezak et al., 2014; Stroth and Eiden, 2010), pituitary (Hirabayashi et al., 2018) and in the adrenal gland (Eiden et al., 2018). In the brain, the greatest accumulation of PACAP-containing cell bodies can be found in hypothalamic and brainstem nuclei. Intensive accumulation of PACAP-immunoreactive (-IR) nerve fibers were observed throughout the hypothalamus, in the amygdaloid and extended amygdaloid complex, in the anterior and paraventricular thalamic nuclei, in the intergeniculate leaflet, in the pretectum, and in several brainstem nuclei, such as the parabrachial nucleus, the sensory trigeminal nucleus, and the nucleus of the solitary tract. The widespread distribution of PACAP in the brain and spinal cord suggests that PACAP is involved in the control of many autonomic and sensory functions as well as higher cortical processes (Hannibal, 2002).

1.1.4 **Stress as a research tool**

Based on the differences in neurochemical responses, two major categories of stressors are recognized, the “physical” or “reactive” and “psychogenic” or “anticipatory” stressors. The first category comprises homeostatic challenges such as changes in cardivascular tone, respiratory distress, visceral or somatic pain, and elevated levels of cytokine or chemokine factors in blood signaling infection or inflammation. The second category covers situations, where the responses are centrally generated in the absence of a physiological challenge. These responses are based on past experiences (memory, context) or are innate by species (fear of predators, heights or open spaces). These responses represent an effort of the organism to prepare a glucocorticoid response in anticipation of, rather than as a reaction to, homeostatic disruption. (Herman et al., 2003). In this context, the experimental stressors used in this work are considered psychogenic, however, the physical component is also present.

1.1.4.1 Restraint

One of most common experimental stressor is the restriction of free movement of an experimental animal (Buynitsky and Mostofsky, 2009). The stressor is mostly psychological stressor in its nature. Two major sub-forms of hypokinetic stress procedures evolved over time; the Immobilization and Restraint. Immobilization is usually achieved by taping the limbs of the animal to a platform (Kvetnansky and Mikulaj., 1970; Marti et al., 2001; Ubeda-Contreras et al., 2018). During Restraint the animal is placed to a restrainer which prevents movement. Nowadays plastic tubes equipped with ventilation holes are mostly used as restrainers, but mesh wire or other types of restrainers were used in the past. The rat or mouse is placed into the restrainer, which does not allow the animal to turn around (Buynitsky and Mostofsky, 2009; Zimprich et al., 2014). Both types of stressor produce appropriate neuroendocrine response, including elevated ACTH, corticosterone and catecholamines (Garcia et al., 2000; Jeong et al., 2000; Kvetnansky et al., 1979) and activation of the respective brain areas (Cullinan et al., 1995; Ubeda-Contreras et al., 2018). Immobilization usually elicits stronger and longer lasting response of stress hormones than restraint and is thus considered a more severe stressor (Marti et al., 2001). However, immobilization is more complicated to perform and experience and skill are needed to prevent animals from self-injury (Ubeda-Contreras et al. 2018). Restraint stress is easier to apply with minimal risk of injury in both rats and mice (Buynitsky and Mostofsky, 2009; Zimprich et al. 2014).

1.1.4.2 Elevated platform

Being exposed to brightly lit, open spaces is considered a stressor for mice and rats, considering their natural habitat and inherent time of activity. When placed on an elevated platform, rats manifest neuroendocrine and behavioral signs of stress (Degroot et al., 2004; Xu et al., 1997, 1998). Exposure to elevated platform inhibits long-term potentiation (LTP) in hippocampus and blocks the LTP in basolateral amygdala-prefrontal cortex pathway (Maroun and Richter-Levin, 2003).

1.1.4.3 Social defeat

Together with crowding, maternal separation, social isolation, chronic subordination, social instability, the social defeat belongs to the category of psychosocial stressors. Disruption of social hierarchy is potent and ethologically relevant stressor and thus bears potential for translation research between rodents and humans (Chaouloff, 2013). Indeed, social conflicts are accompanied

by sympathetic activation and increase of heart rate, blood pressure and body temperature in both rodents and primates (Miczek et al., 2008). Klaus Miczek was one of the first introducing Social defeat in rodents as an experimental stressor (Miczek, 1979). The social defeat, sometimes called the resident-intruder, is based on the observation, that adult male rodents have a strong motivation to defend their territory against unfamiliar males. Therefore when a conspecific intruder is introduced to resident's cage, he is defeated and displays submissive postures (Figure 6) (Miczek et al., 2004). Older, heavier and sexually experienced males, who protect their territory, are selected as residents. This is often strengthened by keeping resident's bedding unchanged for a week prior interaction with intruders (Chaouloff, 2013; Hammels et al., 2015). The interaction between resident and intruder results in elevated ACTH, glucocorticoids, blood pressure and heart rate (Miczek et al., 2008). However the recovery in losers (intruders) is much longer than in winners (residents) probably due to lack of control of the situation by intruders (Koolhaas et al. 2011). The insufficient control over situation could be an explanation for observed lack of adaptation of cardiovascular responses to repeated defeats (Sgoifo et al., 2001; Tornatzky and Miczek, 1993). The physical interaction between resident and intruder must be carefully monitored by the researcher and terminated if there is a risk of serious injury. To prolong the stressor, resident and intruder are separated by mesh or perforated Plexiglas partition, thus animals stay in olfactory, visual and auditory contact, but the risk of injuries is eliminated (Hammels et al., 2015). To further decrease the possibility of habituation in chronic defeat experiments, the intruder is faced with new resident every day (Berton et al., 2006).

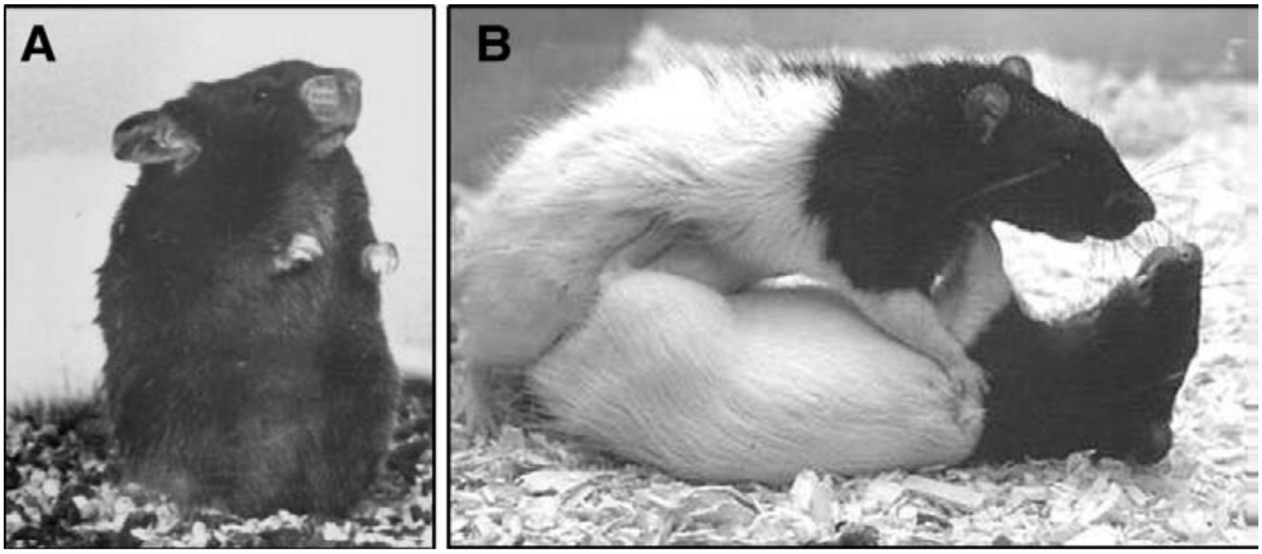

Figure 6. (a) The characteristic defeat posture by an intruder mouse that has been attacked by a resident. (b) The submissive-supine posture by an intruder rat as displayed in reaction to an aggressive posture by an aggressive resident rat. From Miczek et al. 2004

1.1.4.4 Forced Swimming:

Forced swimming (FST) was first introduced by Porsolt et al., (1977) and shown to be sensitive to antidepressants. Later, the application of FST in research was focused on investigation of the coping strategies when facing inescapable stressor (Molendijk and deKloet, 2019). However, swimming alone can be used as stressor because forced swimming elicits robust secretion of stress hormones (Abel, 1993; Rittenhouse et al., 2002). Since swimming has strong physical component there were discussions whether FST has to be considered physical or psychological stressor. Based on studies comparing activation of immediate early genes in brain, forced swimming is considered as primarily psychological stressor, as c-fos activation pattern is similar to restraint and white noise stress (Dayas et al., 2001). However, the physical component has also to be taken to account, especially as there is strong adaptation of the metabolic and neuroendocrine response to repeated swimming (Koolhaas et al., 2011).

1.1.4.5 Duration, adaptation, sensitization and combination of stressors

When a stressor, which is not inherently harmful, persists for a longer time, it is beneficial for the organisms to adapt by decreasing the response of the HPA axis (Grissom and Bhatnagar, 2009). Organisms adapt to repeated homotypic stressors by decreasing neuroendocrine and autonomic readings of stress response in habituation-like manner (Benini et al., 2019), although the adaptation of the HPA axis to repeated stressor does not seem to match all criteria for

habituation (Benini et al., 2019; Rabasa et al., 2015). However, even if the organism is adapted to certain homotypic stressor, the exposure to a novel stressor will induce disproportionately large HPA axis stress response as compared to acutely stressed controls (Herman, 2013). This phenomenon is called sensitization and maintains response flexibility to new threats. Distinct brain areas are involved in adaptation and sensitization processes (Herman, 2013) (Figure 7). Sensitization can also be induced by the exposure to single severe stressor such as immobilization or footshock (Belda et al., 2008; 2012; Rabasa et al., 2015). The adaptation or sensitization depends on several factors including severity of the stressor, individual coping capacity and the predictability and controllability of the stressor (Koolhaas et al., 2011). Therefore in some experimental setups a combination of stressors is used in order to avoid habituation to repeated stressors and to elicit stronger stress response (Ilin and Richter-Levin, 2009; Tsoory and Richter-Levin, 2006).

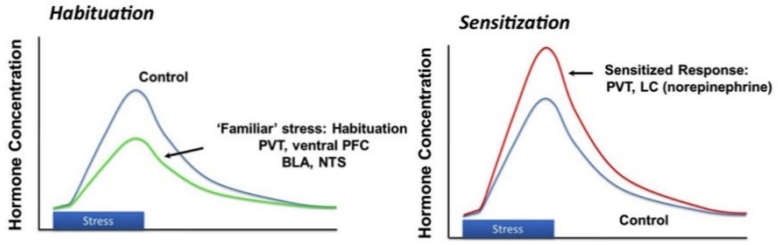

Figure 7. Stress habituation and facilitation. Repeated exposure to the same stressor results in progressive diminution of response magnitude, thought to be mediated by structures such as the prefrontal cortex (PFC) and paraventricular thalamus (PVT). Exposure to a new stressor after either homotypic or hetertypic stressors causes a larger than normal ("sensitized' or "facilitated' response), which may be mediated by enhanced drive from the basolateral amygdala (BLA), PVT or locus coeruleus. From Herman 2013.

1.1.5 **Animal model for studying innate differences in stress reactivity**

When studying biological phenomena it is often advantageous to use experimental animals that show some „abnormality" in studied characteristics. This is also the case of two rat strains differing in the reactivity to stress, the hypo-responsive Lewis (LEW) rats and the hyper-responsive Fischer 344 (F344) rats. The F344 and LEW rats are histocompatible inbred strains that provide a comparative model for investigating interactions between nervous, endocrine and immune systems (Dhabhar et al., 1993). The difference between F344 and LEW rats became apparent in experiments with immunological challenges. LEW rats are known for their susceptibility to experimentally induced arthritis, as they fail to exhibit glucocorticoid-induced immunosuppression to inflammatory stimuli, caused by blunted activity of hypothalamo-pituitary-adrenal (HPA) axis (Sternberg et al., 1992; Wilder et al., 1987). On the other hand, F344 rats showed resilience to

experimentally induced arthritis at the cost of hyper-reactive HPA axis. The response of the HPA axis to stressor is higher in F344 rats compared to other rat strains (Armario et al., 1995; Dhabhar et al., 1993; Herman et al., 1999). Together F344 and LEW rats are used for studying differences in HPA axis reactivity. It is well established, that F344 rats has greater HPA axis response to various forms of acute (Dhabhar et al., 1995, 1997; Moncek et al., 2001; Sternberg et al., 1992), as well as chronic stressors than LEW rats (Dhabhar et al. 1997, Ergang et al., 2015, Vodička et al., 2020). The differences in stress reactivity are interesting in context of other stress-related diseases, therefore F344 and LEW rats are used in research of behavioral aspects of stress-reactivity on drug addiction, (Cadoni, 2016; Kosten and Ambrosio 2002) and anxiety-related disorders, such as Post-traumatic stress disorder (Cohen et al., 2006). Moreover, F344 rats are often used in studies of aging (Mabry et al. 1995; Gardner et al., 2020).

1.2 Connection between gut, brain and HPA axis

It has long been recognized that stress affects the digestive system. However, in recent years, evidence is highlighting the bidirectional communication between gut and brain. Moreover, the digestive system, as well as other surfaces of the organism, is colonized by commensal microorganisms, which interact with mucosal cells and can influence the immune system (Belkaid and Naik, 2013). The gut microbiota play a substantial role not only in the regulation of intestinal physiology, but participate in complex physiological regulation, where the (microbiome)- gut-brain axis provides a bidirectional homeostatic pathway of communication, which includes the autonomic nervous system, enteric nervous system, neuroendocrine signaling pathways and neuroimmune systems (Grenham et al., 2011).

The gut microbiota are forming a complex ecosystem of microorganisms that consists mainly of bacteria, but also yeast, archaea, viruses, fungi and parasites may be present (Gaci et al., 2014; Scarpellini et al., 2015; Williamson et al., 2016; Zoetendal et al., 2006). It is usually stated, that there are more than 10^{13}-10^{14} microorganisms in human gut (Dinan and Cryan 2012; Ley et al. 2006; Savage, 1977; Sekirov et al., 2010), but these numbers have been revised down lately (Sender et al., 2016). Bacteriodetes and Firmicutes are the most abundant bacterial phyla in human as well as in murine gut (Ley et al., 2006). The individual composition of microbiota depends on diet (De Filippo et al., 2010), genetic factors and age (Dinan and Cryan, 2017; Lozupone et al., 2012). Disturbances in microbial community can have impact on whole body homeostasis and even on the brain. The first and well known link are comorbidities in gut and brain diseases. For example,

patients suffering with irritable bowel disease are at higher risk of anxiety or depression (Choi et al., 2019). Similarly, altered microbiota have been implicated in pathophysiology of brain-related disorders such as depression (Valles-Colomer et al., 2019, Parkinson disease (de Vos and de Vos, 2012) and autism (Mayer et al., 2015; Sekirov, 2010). Potential routes of communication between microbiota, gut and brain will be discussed in next chapter.

1.2.1 **Gut-brain communication**

The gut is a highly innervated organ. For instance, there are 200 to 600 millions of neurons in the human enteric nervous system (ENS), which is equal to the number of neurons in the spinal cord (Furness 2006). The neurons are organized into ganglions and plexuses, the submucosal (Meissner's) plexus and the myenteric (Auerbach's) plexus forming complex neural circuits. The ENS is capable of independent regulation of basic gastrointestinal functions, motility, mucous secretion, and blood flow. Central control of gut functions is provided by vagal and, to a lesser extent, spinal motor inputs that serve to coordinate gut functions with the general homeostatic state of the organism (Mertz, 2003; Furness, 2006). The afferent neurons that innervate the gut are divided into extrinsic (spinal and vagal afferents) as well as several classes of intrinsic, primary afferents. Both intrinsic and extrinsic primary afferents show mechano- and chemosensitivity to both physiological and noxious mechanical stimuli. Both extrinsic and intrinsic primary afferents provide input to multiple reflex loops to optimize gut function and maintain gastrointestinal homeostasis during internal perturbations (Mayer, 2011).

The majority of signals from the gut are transduced to the brain by the vagus nerve. Vagus nerve is a principal component of parasympathetic nervous system and is composed of 80% of afferent and 20% of efferent fibers. All layers of the digestive wall are innervated by vagal afferents; however, vagal nerve endings are not in direct contact with microbiota, because the nerve endings do not cross the epithelial layer (Bonaz et al., 2018). The contact with gut lumen is mediated by enteroendocrine cells that release gut hormones (i.e., cholecystokinin, GLP-1, peptide YY, ghrelin, orexin, serotonin etc.) and gut immune cells that release immune-related signaling molecules (cytokines, histamine) to activate receptors on vagal afferents (Mayer 2011). Vagal afferents can also detect some microbial products such as butyrate (Lal et al. 2001) and lipopolysaccharides (LPS) (Gaykema et al., 1998) directly by their own receptors.

The majority of vagal sensory afferents projects to the NTS and it was shown, that infection by pathogenic bacteria *Campylobacter jejuni* increased c-fos expression in the NTS and vagal ganglia (Gaykema et al., 2004, Goehler et al., 2005). NTS has bidirectional connections with PVN and receives input from many limbic areas including amygdala and infralimbic cortex (Ulrich-Lai and Herman, 2009), therefore it is important not only for sensing the inner homeostasis and coordination of autonomic stress response, but it also affects the response of the HPA axis (Herman, 2018). Experiments with vagotomy showed that probiotic treatment reduced anxiety-like behavior and HPA axis response in mice with experimental colitis and this effect was dependent on vagal integrity (Bravo et al., 2011; Bercik et al., 2011a). On the other hand, changes in behavior and hippocampal expression of brain-derived neurotrophic factor induced by antibiotic treatment were not abolished by vagotomy (Bercik et al., 2011b). These data imply the importance of other routes of communication between gut and brain. As mentioned above, the enteroendocrine cells express receptors for microbial products and can produce many hormones and neurotransmitters. Similarly, some bacterial strains are able to produce hormones and signaling molecules (Clarke et al., 2014; Strandwitz, 2018) and thus increase their plasma levels. Although most of the hormones and signaling molecules do not cross the blood brain barrier (BBB), still they may influence the central nervous system by influencing organs outside the BBB (such as pituitary gland, immune organs, kidney, adrenals etc.) (Clarke et al., 2014). Many of these molecules can also reach the brain and partially exert their effects via circumventricular organs (CVOs), where the BBB is reduced and which contain sensory receptors for many of these signaling molecules including LPS, glucocorticoids, prostaglandins etc. (Sisó et al., 2010). Gut microbiota also have a profound effect on tryptophan metabolism. As much as 95 % of body serotonin is produced in gastrointestinal system and microbiota alter metabolic pathways of 5-HT precursor tryptophan towards the kynurenine pathway and thus influence not only the tryptophan availability, but also products of kynurenine pathway, which may affect the CNS (Kennedy et al., 2017).

70-80 % of body immune cells are located in the gut-associated lymphatic tissue. The immune cells are relatively hypo-responsive to commensal bacteria, but maintain responsiveness under pathological conditions. Vagal afferents in the proximity of mucosal immune cells contain receptors for signaling molecules (proteases, histamine, serotonin, CRH and cytokines) produced by immune cells in Peyer's patches and cells within gut epithelium (Mayer et al., 2011). Cytokines can also enter the brain via CVOs and/or through BBB via specific receptors (Dantzer et al., 2008).

1.2.2 **GF mice model**

There are several tools for studying the role of microbiota in the physiology of the host. Antibiotic treatment, probiotics or mildly pathogen bacteria are typical experimental approaches to alter gut microbiota (Kennedy et al., 2018). However, it is important to be aware of the fact that all of them have some limitations. For example, antimicrobial treatment using antibiotics, does not lead to total depletion of microbiota, but mostly to shift in relative abundance of phyla depending on the antibiotics used (Rakoff-Nahoum et al., 2004). Another possibility to dissect the impact of microbiota on organism's physiology is to study animals without microbiota also known as the germ-free (GF) animals. Mice are the most frequently used GF animals but other organisms including rats, piglets and Drosophila were used. GF mammals are delivered by Caesarean section and kept under sterile condition throughout entire life or can be also generated by breeding GF together. The sterility is regularly checked by Schwabs cultivation and sentinel mice are sacrificed usually every 2 week and examined for bacterial presence.

1.2.3 **GF mice and stress**

Several studies have shown that the HPA response to stress is affected by composition of microbiota. GF mice show exaggerated HPA response to acute restraint stress and this effect can be reversed by monoassociation with probiotic bacteria *Bifidobacterium infantis* and the normalization of stress response was also dependent on age at colonization (Sudo et al., 2004). Exaggerated response of HPA axis to psychological stress was confirmed by us and others (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Vagnerová et al., 2019).

Stress is often regarded as contributing factor for anxiety-related disorders (McEwen et al., 2003), but GF mice do not display anxiety-like behavior. The majority of studies reported decreased anxiety-like behavior in GF mice, but the results depended on strains and tests used (summarized in Luczynski et al., (2016)). The importance of microbiota in anxiety development was nicely illustrated in work from Prof. Bercik's lab. Anxiety-like behavior was induced by maternal separation in SPF mice. In GF mice no anxiety was observed after the same treatment. Interestingly, the anxiety phenotype was induced by colonizing GF maternally separated mice with microbiota from SPF non-separated mice. On the other hand, simply colonizing GF mice with microbiota from maternally separated SPF mice did not lead to anxiety-like phenotype (DePalma

et al., 2015). It had also been shown, that manipulation with microbiota (treatment with antibiotics or probiotics) can alter anxiety-like behavior (Bercik et al., 2011b; Desbonnet et al., 2015; Savignac et al., 2014). Several studies also highlighted microbiome-dependent changes in neurotransmitters and/or their receptors in brain structures involved in the regulation of HPA axis or anxiety. Chronic stress has been shown to alter composition of microbiota, however severe stress protocols have to be applied (Bharwani et al., 2016; Galley et al., 2014; Wong 2016).

2 Aims

It is clear from previous text, that stressors are naturally inseparable from life. However, the individual responses to certain stressors vary greatly. Therefore, it is important to study the stress response and dissect mechanisms of its action in order to improve therapy and prevention of stress-related diseases. The topic of this thesis is focused on the analysis of stress response in animals differing in HPA axis responsivity (F344 and LEW rats and GF vs. SPF mice) with special emphasis on the regulation of HPA axis, glucocorticoid regeneration and influence of gut microbiome. In the first project, we studied the effect of various stress paradigms on the expression of genes encoding proteins involved in central and peripheral regulation of glucocorticoid signaling and in regulation of HPA axis responsiveness using hyper-reactive Fisher 344 and the hypo-reactive LEW rats, which represent two ends of a spectrum of HPA axis responsiveness to stress and vulnerability to immune diseases. The second project was focused on the role of microbiota in shaping stress response. Microbiota are capable of modulating the reactivity of the HPA axis and GF mice show exaggerated response of HPA axis to psychological stressors. Therefore, we focused on interaction between stress and gut microbiota, i.e. how microbiota shape the response of HPA axis to stress. Specifically, the following aims were investigated:

1. The impact of short-term and chronic stress on activation of the HPA axis and glucocorticoid metabolism in the structures of the HPA axis in brain regions participating in HPA axis regulation in stress hyper-reactive Fischer 344 and hypo-reactive Lewis rats.
2. The effect of chronic stress on local metabolism and regeneration of glucocorticoids in lymphoid organs
3. The effect of microbiota on activation of the HPA axis by acute and chronic stress
4. The effect of microbiota and stress on regulatory pathways in the intestine.
5. Assessment of microbiota-dependent differences in behavioral phenotype in resident-intruder paradigm

3 Methodological approaches

The methods are described in detail in enclosed publications.

3.1 Stress procedures

3.1.1 Social defeat

Social defeat paradigm was used in both rat and mice experiments. Specifically, 65-day-old male Fisher 344 rats were used as residents and intruders. Residents were housed individually for one week before the experiment, whereas the intruders were housed in groups of three or four. Following the seven-day isolation period of the residents, the social encounter was performed for seven consecutive days, and arranged to ensure that each intruder rat met each of the corresponding residents for 30 minutes. The animals were sacrificed after the last resident-intruder session. (Publication A). In experiments with F344 and LEW rats we used almost identical protocol with following alteration. The intruder F344 or LEW rat was exposed to older male retired breeder of aggressive Long Evans rats and the confrontation with the resident lasted 15 min once daily for ten consecutive days (Publication E).

In the case of murine experiments two-month-old (GF) and (SPF) male BALB/c mice were used. GF animals were kept under sterile conditions in Trexler-type isolators since birth. One month before the beginning of the experiments, the SPF mice were transferred to similar isolators to ensure identical conditions for all groups during the experiments. Animals were housed in groups of 4–5 per cage. Retired male breeders (7-months to 1-year-old) of the BALB/c strain were used as residents. Resident mice were housed individually for 7 days before the experiment without a change of bedding (to enhance territoriality and aggression). On the days of testing, each intruder was removed from his home cage and placed into the home cage of a resident. Following the 10 min interaction, the mice were divided by a steel mesh to preserve sensory contact between the mice for the next 50 min. Thus, the intruder was subjected to continuous psychological stress due to sensory interaction with the resident. This procedure was repeated for 5 consecutive days with different residents to prevent any habituation to the resident. Following the last stress session, the animals were removed from the isolator and anesthetized with isoflurane vapor (Publication C).

3.1.2 Three-day variable stress protocol (Publication B)

Male F344 and LEW rats that were 60–65 days old at the beginning of the experiments were used On Day 1, the animals were exposed to forced swim for 15 min (water temperature 22 ± 1 °C). On Day 2, the animals were placed on an elevated platform (12 cm × 12 cm at a height of 70 cm above floor level) in brightly lit room for 30 min. This trial was repeated three times, with a 60 min interval between trials. On day 3, the rats underwent a 2-h restraining stress in an opaque plastic box that prevented the free movement of the animal. These protocols were applied simultaneously to all rats in the cage. The rats were sacrificed immediately after termination of the last stressor.

3.1.3 Acute restraint stress (Publication D)

The adult GF and SPF male mice were subjected to a single 2-hour restraint stress in 50-ml conical centrifuge tubes equipped with multiple ventilation holes.

3.1.4 Acute inflammatory stress (Publication F)

Acute colitis was induced in male Balb/c mice (six to seven weeks old) by administering 2% dextran sodium sulfate in drinking water for a five-day period.

3.2 Laser capture microdissection

3.2.1 Brain areas (Publication A and B)

Coronal brain sections (20 µm) were serially cut with a cryostat at −19°C. The sections of the studied structures were mounted onto slides coated with polyethylene naphthalate membrane fixed in 95% ethanol, stained with 4% cresyl violet acetate and washed three times in 95% ethanol. The PVN, central (CeA) and lateral amygdala (LA), prelimbic prefrontal (plPFC) and infralimbic prefrontal cortex (ilPFC), hippocampal CA2 and CA3 regions, and ventral (vCA1) and dorsal (dCA1) parts of CA1 region, were identified based on standard anatomical landmarks and stereotaxic coordinates according to Paxinos and Watson (2007). The studied brain structures were dissected using a LMD6000 Laser Microdissection System and captured into the caps of the microcentrifuge tubes. Microdissected tissues were homogenized in 75 µl RLT buffer (Qiagen, Hilden, Germany) and stored at −80°C until RNA isolation.

3.2.2 Colon and MLN (Publication F)

The 20-µm tissue sections were cut from frozen blocks of the colon and MLN, and transferred to polyethylene-naphtalate membrane slides. The tissues were dehydrated and stained

with cresyl violet acetate and eosin B. Immediately after staining, the tissues were dissected using the Leica LMD 6000 Laser Microdissection System. Staining allowed for the identification of functionally different compartments in the gut (isolated lymphoid follicles (ILF), lamina propria, colonic crypt epithelium (CCE)) and MLN (cortex, paracortex, medulla).

3.3 mRNA expression analysis

Total RNA was isolated using an RNeasy Micro Kit from the captured microsamples and using a GeneElute Mammalian Total RNA Miniprep Kit from macrosamples. Single-strand cDNA was prepared from total RNA isolated from tissue microsamples and macrosamples using random hexamers and either Enhanced Avian Reverse Transcriptase or High Capacity cDNA Reverse Transcription Kit. The cDNA samples were analyzed by real-time PCR using TaqMan Assays specific for the studied transcript

3.4 Hormone measurement

Plasma corticosterone levels were determined by a commercially available Corticosterone rat/mouse ELISA (Publication D) or RIA (Publications A, B and E).

Plasma ACTH levels were determined by ACTH ELISA kit (Publication E)

4 Summary of main results

4.1 Effect of short term and chronic stress on HPA axis activation and local glucocorticoid metabolism in the components of the HPA axis and in brain areas involved in HPA axis control in rat strains differing in HPA axis reactivity

Chronic psychosocial stress upregulated expression of *Hsd11b1* in F344 rats in brain regions involved in HPA axis regulation, notably plPFC, CeA, LA, and CA1 and CA2 hippocampal subfields. On the other hand, stress exposure had no effect on *Hsd11b1* expression in effector regions of the HPA such as the PVN, pituitary, adrenal cortex and adrenal medulla (Vodička et al., 2014/Publication A).

The three-day stress protocol was accompanied with similar pattern of *Hsd11b1* expression as chronic psychosocial stress however the effect was strain-dependent. In F344 rats, the *Hsd11b1* was elevated by stress in CeA, vCA1 and CA2 hippocampal subfields whereas in LEW rats, stress stress upregulated *Hsd11b1* expression in plPFC and LeA. No stress induced changes of *Hsd11b1* expression were observed in canonical components of the HPA axis (PVN, pituitary, adrenal cortex, adrenal medulla). Stress also stimulated the expression of neuropeptides *Oxt*, *Crh*, *Ucn3* and *Pacap* in PVN of both strains but expression of amygdalar *Crh* was elevated only in LEW and *Ucn2*/*Ucn3* in F344 rats, respectively. Stress also upregulated expression of enzymes of adrenal synthesis of catecholamine, the *Th* and *Pnmt*, and this upregulation was more pronounced in F344 rats (Ergang et al., 2015/Publication B).

4.2 The effects of chronic stress on local metabolism and glucocorticoid regeneration in lymphatic organs

Chronic psychosocial stress increased the expression of *Hsd11b1* in mesenteric lymphatic nodes (MLN) and spleen of F344 rats (Ergang et al., 2015/Publication B). Similarly, the identical stress paradigm upregulated the regeneration of corticosterone from 11-dehydrocorticosterone in the thymus, spleen and (MLN) of both F344 and LEW rats. Compared with the F344 strain, the LEW rats showed higher corticosterone regeneration in splenocytes of unstressed rats and in thymocytes and MLN mobile cells of stressed animals but corticosterone regeneration in the stroma of all lymphoid organs was similar in both strains (Ergang et al., 2018/Publication E).

Similarly to psychosocial stressor in rat, the inflammatory stress in mice represented by dextran sulfate sodium induced colitis, led to increased *Hsd11b1* expression in specific

microanatomical compartments of the mucosal immune system. More specifically, colitis increased *Hsd11b1* expression in the colonic crypt epithelium, isolated lymphatic follicles and cortex of MLN cortex but not in the lamina propria of colon and paracortex and medulla of the MLN. Expression of *Hsd11b1* positively correlated with *Tnfα* (Ergang et al., 2017/Publication F).

4.3 Role of microbiota in HPA axis activation in acute and chronic stress

Plasma corticosterone response to acute restraint stress was higher in GF than in SPF mice. In pituitary, acute stress and microbiota downregulated the expression of *Crhr1* and microbiota downregulated *Pomc* expression. Microbiota upregulated expression of genes *Cyp11a1*, *Hsd3b1* and *Cyp21a1* encoding steroidogenic enzymes in adrenals (Vagnerová et al., 2019/Publication D). Chronic psychosocial stress and the absence of microbiota increased expression of regulatory co-chaperon *Fkbp5* in pituitary and expression of adrenal enzymes involved in synthesis of catecholamines *Th* and *Pnmt* (Vodička et al., 2018/Publication C).

4.4 Role of microbiota in glucocorticoid regulation in colon

Both acute restraint stress and microbiota modulated the expression of some steroidogenic genes in colon, especially *Nr5a2*, which encodes the crucial transcriptional regulator of intestinal steroidogenesis LRH-1 and *Hsd3b2*, both genes were decreased by stress and absence of microbiota. Interaction between stress and microbiota was found in expression of *Cyp11a1*, and *Hsd3b1;* genes encoding steroidogenic enzymes in colon (Vagnerová et al. 2019/Publication D). Chronic psychosocial stress downregulated the expression of *Hsd11b1* and dampened the expression of a panel of cytokines depending on the presence or absence of gut microbiota (Vodička et al. 2018/Publication C).

4.5 Assessment of behavioral changes in relationship to microbiota in chronic psychosocial stress

GF intruder mice spent less time in total defensive behavior during interaction with residents. This effect was mainly caused by escape/flight behavior. No difference in offensive behavior was found between GF and SPF residents (Vodička et al. 2018/Publication C).

5 Discussion

This thesis is focused on the effect of stress on regulation of the HPA axis and regeneration of glucocorticoids. The first section is focused on the effects of stress on mRNA expression of enzymes involved in glucocorticoid regeneration and selected neuropeptides linked to HPA axis regulation in the regulatory brain areas as well in the peripheral parts of the HPA axis in stress hyper-reactive F344 and stress hypo-reactive LEW rats. The second section is dedicated to dissecting the role of microbiota in shaping the response in peripheral components of the HPA axis in SPF and GF mice challenged to acute and chronic stressors. The third section addresses the effects of various stressors on local glucocorticoid metabolism in peripheral organs with respect to HPA axis reactivity.

5.1 The effect of various stress paradigms on expression of genes involved in central and peripheral regulation of glucocorticoid signaling and HPA axis regulation in stress hyper-reactive F344 rats and stress hypo-reactive LEW rats

5.1.1 The impact of chronic psychosocial stress on mRNA expression of the enzyme 11β-hydroxysteroid dehydrogenase type 1 in the components of the HPA axis and in brain areas in stress hyper-reactive Fischer 344 rats.

The first study (Vodička et al., 2014/Publication A), was focused on effect of chronic psychosocial stress on *Hsd11b1* expression in brain areas involved in HPA axis regulation in F344 rats. We evaluated the effect of repeated psychosocial stressor on expression of enzymes regulating local concentration of glucocorticoids and peptides associated with the regulation of the HPA axis in stress hyper-reactive F344 rats in principal components of the HPA axis and in brain areas involved in the HPA regulation. Disruption of social hierarchy by repeated resident-intruder paradigm resulted in increased physiological stress markers (plasma corticosterone, ACTH, *Crh* expression in the PVN) in both residents and intruders. All stress markers were significantly higher in residents compared to intruders. Additionally, intruders spent less time displaying social behaviors compared to residents.

The enzyme 11HSD1 acts to increase active form of glucocorticoids intracellularly due to conversion of inactive 11-oxo derivatives of glucocorticoids to active hormones. The expression of 11HSD1 was previously detected in the PVN, anterior pituitary, adrenal glands and limbic brain regions (Sakai et al., 1992; Shimojo et al., 1996, Hanafusa et al., 2002). Therefore, we were

interested whether the expression of *Hsd11b1* is affected by repeated social stress. Glucocorticoids act as feedback inhibitors on HPA axis, but several days of social stress did not have an effect on *Hsd11b1* expression in either PVN, pituitary, adrenal cortex or adrenal medulla. Adrenal medulla is not a part of HPA axis, but glucocorticoids are essential for epinephrine production by chromaffin cells (Zuckerman-Levin et al. 2001). On the other hand, social interaction between resident and intruder increased expression of *Hsd11b1* in central and lateral nuclei of amygdala, prelimbic cortex and vCA1 and CA2 fields of the hippocampus, i.e. in brain areas activated by psychological stressors and associated with HPA axis regulation (Ulrich-Lai and Herman, 2009; deKloet at al., 2005). Presented data are in line with the known role of limbic structures in HPA axis regulation, which is, at least partially mediated by glucocorticoids. Prelimbic cortex inhibits the HPA axis (Herman et al., 2003) and its stimulation is sufficient to trigger the inhibition of HPA axis response to psychogenic stress (Jones et al. 2011). GR knockdown in this region leads to increased HPA responses to acute stress (McKlveen et al., 2013).

Amygdala plays an important role in glucocorticoid-mediated regulation of the HPA axis. Corticosterone application to amygdalar region prolonged HPA axis response (corticosterone) to single stressor (Shepard et al., 2003). Interestingly, the overexpression of MRs in basolateral amygdala led to reduced glucocorticoids secretion after acute stressor and decreased anxiety (Mitra et al., 2009a). The high-affinity MRs are heavily occupied during basal conditions, whereas low-affinity GRs are heavily occupied only by stress levels of glucocorticoids, suggesting the importance of local glucocorticoid modulation by 11HSD1.

The hippocampus exerts predominantly an inhibitory tone on the HPA axis response and expresses high levels of glucocorticoid receptors (deKloet et al., 1998). The increase of *Hsd11b1* following stress in the vCA1 and CA2 regions of the hippocampus is in agreement with previously published study showing the effect of arthritic stress in rats on the whole hippocampus (Low et al., 1994) but not with the effect of chronic psychosocial stress on hippocampus in tree shrews (Jamieson et al., 1997). This difference may underlie not only in species-specific differences in control of 11HSD1 but also in the time and duration of stress applied. It was previously shown that intact hippocampal cells show reductase activity and thus reactivate inert 11-dehydrocorticosterone to corticosterone and this activity was inducible by glucocorticoid excess (Rajan et al., 1996). The increased expression of *Hsd11b1* after social stress might increase the glucocorticoid signal in

hippocampus and enhance the inhibitory effect of hippocampus on PVN. It is known that the hippocampus can be functionally divided to dorsal and ventral portion, where dorsal part is primarily involved in cognitive functions, whereas ventral part is associated with emotional control (Faneslow and Dong, 2010). Nevertheless it is problematic to match the distinctive changes of *Hsd11b1* expression observed in hippocampal subfields to specific hippocampal functions after social stress. However, it seems that the inhibition of HPA axis is at least partially mediated via ventral hippocampus, since bilateral lesion of ventral subiculum increased response to psychological stressors (Herman et al., 1998) and corticosteroids have been shown to act as structural and functional modulators of limbic areas, including learning and memory (deKloet et al., 2005; Herbert et al., 2006). Our findings suggest that upregulation of *Hsd11b1* and amplification of the glucocorticoid signal might be a relevant mechanism in feedback regulation of stress responses in limbic structures.

The glucorticoid signal in brain is largely conveyed via intracellular receptors. The MRs have high affinity for corticosterone and thus are occupied even if glucocorticoid concentration is low. Lower affinity GRs are occupied when glucocorticoid levels are elevated, for example at circadian peaks of plasma glucocorticoids or during stress (deKloet et al. 2005, Herbert et al., 2006). Therefore, we studied the effects of chronic psychosocial stress also on the expression of *Nr3c1* encoding the GR. This expression was not affected by chronic psychosocial stress in any of examined brain areas except for vCA1 of hippocampus. Minimal changes in *Nr3c1* expression together with elevated expression of *Hsd11b1* in structures crucially involved in the regulation of stress-related behavior and modulation of hippocampal functions (Herbert et al., 2006; Ulrich-Lai and Herman, 2009), suggest that adaptive reaction of these limbic structures to chronic psychosocial stress is based rather on the changes in 11HSD1 than GR expression.

Interestingly, stress does not elevate expression of *Hsd11b1* in PVN, pituitary or adrenal glands, the principal components of the HPA axis, although all of these structures express 11HSD1. Collectively, it can be assumed that (1) the upregulation of *Hsd11b1* in prelimbic cortex, amygdala and some hippocampal fields might enhance the glucocorticoid signal by converting 11-dehydrocorticosterone to corticosterone, and (2) lack of changes in *Nr3c1* expression suggests that local modulation of glucocorticoid feedback signal in limbic areas is conveyed by 11HSD1 rather than GR.

5.1.2 **The effect of three-day variable stress on expression of *Hsd11b1* and peptides involved in regulation of the central and peripheral parts of the HPA axis in the stress hyper-reactive F344 rats and stress hypo-reactive LEW rats**

The second study (Publication B/Ergang et al., 2015) was aimed at the differences between the stress hyper-reactive F344 rats and the stress hypo-reactive LEW rats in short-term stress protocol. This combination of three different stressors within three day was originally designed to mimic traumatic events in early life as it elicits strong stress response and minimizes habituation (Tsoory et al., 2006; Ilin and Richter Levin, 2009). We applied this protocol to assess the effect of stress on HPA axis response in the periphery and to evaluate mRNA expression of genes encoding 11HSD1 and neuropeptides CRH, UCN2 and UCN3, OXT and PACAP in the brain regions modulating the HPA axis activity.

As expected, the HPA axis was activated in both strains after 3 days of variable stress. At the end of the last stress session the F344 rats had greater corticosterone levels compared to LEW counterparts. It is difficult to categorize the three-day variable stress protocol in terms of acute or chronic. Regardless of stress duration, previous studies showed higher corticosterone levels, indicating activation of HPA axis in F344 compared to LEW rats following both acute and chronic stress regimes (Dhabhar et al., 1993; 1997; Elenkov et al., 2008; Moncek et al., 2001; Sternberg et al., 1989) and our study was in accordance with these studies. Similarly, the expression of enzymes crucial for catecholamine synthesis in adrenal medulla, the *Th* and *Pnmt* (Kvetnansky et al., 2009), was upregulated by stress regime and this upregulation was more pronounced in F344 rats, which corresponded to elevated plasma catecholamines in F344 rats following stress compared to LEW rats (Elenkov et al., 2008).

The stress protocol also upregulated the expression of *Hsd11b1* in brain areas involved in the regulation of the HPA axis (deKloet et al., 2005; Ulrich-Lai and Herman, 2009). On the other hand, three-day variable stress protocol did not upregulate the expression of *Hsd11b1* in the main components of the HPA axis (PVN, pituitary and adrenals), which is in agreement with our previous study using psychosocial stress (Vodička et al., 2014/ Publication A). Distinct areas of the prefrontal cortex, amygdala and vCA1 and CA2 regions of the hippocampus were activated in strain-specific manner because the application of stressors led to increased expression of *Hsd11b1* in CeA, vCA1 and CA2 of F344 rats and in prelimbic PFC, infralimbic PFC, and LA of LEW rats.

The strain-dependent changes might be related to several factors such as ceiling effect in F344 rats and the role of specific brain areas in glucocorticoid feedback regulation. LEW rats had lower basal *Hsd11b1* expression in the prefrontal cortex, vCA1 and CA3 than the F344 strain. Stress never stimulated *Hsd11b1* expression in the brain of LEW rats to a higher level than in stress-stimulated F344 strain. The prefrontal cortex plays an important role in inhibiting the HPA axis and this effect is, at least to some extent, mediated by glucocorticoids as corticosterone implantation to medial PFC reduced glucocorticoid secretion after stress (Diorio et al. 1993). A more recent study investigating particular parts of the prefrontal cortex has shown that GR knockdown confined to the ilPFC caused acute stress hyper-responsiveness, sensitization of stress responses, whereas GR knockdown in plPFC increased hypothalamic-pituitary-adrenocortical axis responses to acute but not chronic stressors (McKlveen et al., 2013). High basal *Hsd11b1* expression in ilPFC of F344 rats together with the inability of F344 rats to further increase *Hsd11b1* expression in stress and increase glucocorticoid signal may lead to insufficient activation of prefrontal inhibitory feedback loop leading to increased activity of the HPA axis. Together with GRs in prefrontal cortex, GRs in amygdala and hippocampus are also involved in HPA axis regulation (Herman et al., 2003). It has been shown that prefrontal, amygdalar and hippocampal GRs are necessary for negative feedback after both mild and robust acute psychogenic stressors but not after hypoxia, a systemic stressor (Furay et al., 2008).

We observed differences in hippocampal *Hsd11b1* expression between F344 and LEW rats; stress induced upregulation of *Hsd11b1* in vCA1 and CA2 subfields of the hippocampus only in F344 rats. Hippocampus is important for regulation of the HPA axis response and ventral pole of the hippocampus plays a prominent role as it is involved in HPA axis inhibition and processing of anxiety, due to its abundant connection with amygdala and prefrontal cortex (Fanselow and Dong, 2010). In addition, the response of the ventral part of the hippocampus to glucocorticoids differs from that of the dorsal hippocampus (Maggio and Segal, 2009). The possibility that increased hippocampal *Hsd11b1* expression may be relevant to stress regulation can be supported by several findings. First, the ratio of corticosterone/11-dehydrocorticosterone can dynamically change in the brain cortex, amygdala and hippocampus (Cobice et al., 2013). Second, experiments with hippocampal explants demonstrated that intact hippocampal cells reactivates inactive 11-dehydrocorticosterone to active corticosterone (Rajan et al., 1996). In summary, the three-day stress protocol induced the upregulation of *Hsd11b1* in the majority of examined brain regions but

only sporadic changes in expression of *Nr3c1*. Thus, we can hypothesize that stress might intensify the glucocorticoid signal in limbic structures mainly due to the conversion of local 11-dehydrocorticosterone to corticosterone but not via upregulation of GR.

On the other hand, we did not observe any stress-induced elevation of *Hsd11b1* in the main components of the HPA axis: the PVN, pituitary and adrenal glands either in F344 or LEW rats. These results extend our previous study, where we observed similar pattern after chronic psychosocial stress in F344 rats (Vodička et al., 2014). Even though we did not find any changes to stress, we demonstrated strain-dependent differences in *Hsd11b1* expression. The lower pituitary expression of *Hsd11b1* in F344 rats was accompanied by lower expression of *Nr3c1* gene encoding the GR, which might contribute to differences in glucocorticoid negative feedback on HPA axis between F344 and LEW rats (Gomez et al., 1998; Simar et al. 1997). We found strain-specific differences in expression of *Hsd11b1* and *Hsd11b2* in adrenal cortex and in the case of *Hsd11b2* also in adrenal medulla. Increased 11HSD1 in adrenal cortex of F344 rats could be one of the factors behind ACTH-independent elevation as a response to novel stressor following prolonged stress (Dhabhar et al., 1997). Although it has been previously demonstrated that inhibitors of 11HSDs in adrenals reduced the expression of the glucocorticoid-dependent enzyme PNMT in adrenal medulla (Shimojo et al., 1996), we did not observe any stress-induced effect on *Hsd11b1* and *Hsd11b2* expression in adrenal medulla. Moreover, the expression of glucocorticoid-independent *Th* and glucocorticoid-dependent *Pnmt* in our experiment showed similar expression pattern, therefore we consider any intracrine regulation being unlikely. Stress also upregulated expression of 11HSD2 in adrenal cortex in both strains and this upregulation was more pronounced in F344 rats. This finding is in line with previous work showing higher adrenal corticosterone level in stressed LEW than in stressed F344 rats (Moncek et al., 2001).

The extent of the stress response depends also on the activity and appropriate regulation of the CRH signaling system, which has been well described both in vitro and in vivo. Nevertheless, the data regarding central regulation of CRH pathway in F344 and LEW rats are scarce. In the PVN the three-day stress upregulated the expression of *Crh* and *Oxt* similarly in both strains. In contrast, *Crh* expression in amygdala was higher in basal conditions in stress hyper-reactive F344 rats than in LEW rats and stress led to increased expression of *Crh* only in LEW rats. This observation is in agreement with the previous study showing that overexpression of *Crh* in CeA is associated with

HPA axis hyperactivity (Flandreau et al., 2012). We have shown that expression of other members of the *Crh* family in the brain, *Ucn2* and *Ucn3*, is region- and strain-specific. In the PVN, stress increased in both strains the expression of *Ucn3*, but not *Ucn2*, whereas in amygdala stress upregulated both *Ucn2* and *Ucn3*, but only in F344 rats. It was previously shown that glucocorticoids upregulate UCN2 expression (Chen et al., 2003; Tillinger et al., 2013) and thus we can hypothesize the higher stress-induced glucocorticoid levels, together with upregulation of *Hsd11b1* in CeA of stressed F344 rats might reinforce the stress-dependent elevation of *Ucn2* in this strain. The expression of CRH in the PVN can also be modulated by the neuropeptide PACAP (Stroth et al., 2011). The *Pacap* expression was upregulated by stress in the PVN and LA, but not in CeA in both strains and similar expression pattern was previously observed in Sprague-Dawley rats after chronic variable stress (Hammack et al., 2009). The effects of studied neuropeptides depend on their receptors, therefore we looked for stress-related changes in mRNA expression of *Pac1*, the receptor for *Pacap*, the receptor *Crhr1*, which has the highest specificity for *Crh,* and *Crhr2* with higher specificity for *Ucn2* and *Ucn3*. Our stress paradigm increased expression of *Pac1* in CeA, LaA and PVN and expression of *Crhr2* in the PVN and decreased expression of *Crhr1* in pituitary; all changes were observed in both strains. In the case of pituitary *Crhr1* and *Pac1* we found also the effect of strain. We did not observe changes in *Crhr1* expression in amygdala and did not detect *Crhr2* by our method. Increased CRHR2 expression in PVN without changes in paraventricular and amygdalar CRHR1 was reported previously (Zohar and Weinstock, 2011). We observed stress-induced upregulation of *Pac1* in amygdala, which is in contrast to study of Hammack et al. (2009) who found no changes in *Pac1* expression in amygdala after one week of chronic variable stress. This discrepancy may be related to longer stress protocol, because stress-induced upregulation of PAC1 was found in "extended amygdala" in bed nucleus of the stria terminalis (Hammack et al., 2009).

5.2 The role of microbiota in shaping stress response

The second project was aimed at environmental factors influencing the HPA axis. The microbiota are known to modulate neuroendocrine, immune and behavioral response of the organisms. The GF and SPF mice were challenged with chronic psychosocial or acute restraint stressor and responses of genes involved in regulation of glucocorticoids were determined.

5.2.1 **The influence of microbiota on expression of genes participating in HPA axis regulation in chronic psychosocial stress**

The first study (Vodička et al., 2018/Publication C) was focused on the determination how gut microbiota may affect behavior and HPA axis reactivity during the exposure to repeated social defeat. The commensal microbiota affect brain functioning, emotional behavior and ACTH and corticosterone responses to acute stress, therefore we focused our attention on how microbiota shapes behavioral, HPA axis and gut responses in chronic social defeat stress. We observed distinct behavioral profiles in GF and SPF mice subjected to chronic resident-intruder stress. The GF mice showed less total defensive behavior than SPF mice and this difference was caused mainly by difference in escape/flight behavior. Escape behavior might be provoked by higher aggression of residents, therefore we compared GF and SPF residents and found no differences in offensive behavior. No studies have compared behavior of GF and SPF mice in resident-intruder paradigm yet, but it has been demonstrated that germ-free status can modify social preference in mice (Arentsen et al., 2015, Desbonnet et al., 2014). On the other hand, many studies focusing on anxiety-like behavior have been carried out and majority of them observed diminished anxiety behavior in GF mice (Arentsen et al., 2015, Clarke et al., 2013, Crumeyrolle-Arias et al., 2014, De Palma et al., 2015, Diaz Heijtz et al., 2011, Neufeld et al., 2011, Nishino et al., 2013). In our experimental setup the escape/flight behavior can be classified as anxiety-like behavior, suggesting that GF mice exhibit less anxiety-like behavior in repeated psychosocial stress.

We observed multiple stress and/or microbiota dependent changes in HPA axis. Stress increased expression of *Pomc* but not *Crhr1* and microbial status did not have any effect on *Pomc*, and *Crhr1* in the pituitary. Our data agree with previous observation demonstrating that chronic immobilization for 8 and 15 days increased pituitary *Pomc* expression (Rabadan-Diehl et al., 1996). The absence of any effect of repeated psychosocial stress on *Crhr1* is in line with our previous report in analogous stress paradigm in F344 rats (Vodička et al., 2014). Corresponding results are reported in Raone et al. (2007), where repeated unavoidable stress exposure did not lead to changes in pituitary CRHR1 (Raone et al., 2007).

It has been shown repeatedly, that absence of microbiota enhances the HPA response to psychological stressors (Ait-Belgnaoui et al., 2012; Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Sudo et al., 2004). The question remains, where this difference is located. Some authors

focused on GF and SPF mice differences in neurochemistry of brain areas involved in HPA axis regulation (Clarke et al., 2013, Neufeld et al., 2011, Sudo et al., 2004). The second possible mechanism is alteration of negative feedback in the HPA axis. Although we found no significant effect of microbiota in pituitary *Crhr1* and *Pomc*, the expression of *Fkbp5* was upregulated in the GF mice. FKBP5 acts as a co-chaperone that exerts an inhibitory role on GR signaling (Bekhbat et al., 2017) and therefore it is possible that higher *Fkbp5* expression in the pituitary gland of GF mice might decrease efficiency of the negative feedback via GR and contribute to increased HPA activity in GF mice observed by others. This possibility is further supported by findings that, the expression of *Fkbp5* and the cytoplasmic level of GR are elevated by mild chronic stress in rats (Guidotti et al., 2013), and that the expression of *Fkbp5* in mice was augmented by chronic treatment with corticosterone (Lee et al., 2010). The FKBP5 protein represents fast inhibitory feedback loop and is directly activated by corticosterone. Considering the fact that taking mice out of the isolator is a stressful procedure, elevated basal *Fkbp5* expression might reflect higher response of GF mice to this unavoidable handling stress.

Even though is known that microbiota play a role in shaping emotionality, regulation of brain neurochemistry and HPA axis response, we were the first showing the effect of microbiota on adrenal glands under the conditions of repeated psychosocial stress. Our result show that the GF status is associated with upregulation of genes encoding key proteins involved in glucocorticoid and catecholamine synthesis in adrenal gland. On the other hand, stress affected only genes involved in the synthesis of epinephrine, but not corticosterone synthesis. These findings are in line with previous report showing the absence of the chronic subordinate colony housing, another form of psychosocial stress, on expression of key steroidogenic enzymes StAR and CYP11a1 (Uschold-Schmidt et al., 2012). By contrast, microbiota was shown to affect degradation and biosynthesis of catecholamines in brain, but available results are contradictory (Crumeyrolle-Arias et al., 2014; De Palma et al., 2015; Diaz Heijtz et al., 2011; Nishino et al., 2013) and this makes comparisons between the brain and the adrenal gland difficult.

Despite the fact, that glucocorticoids are heavily involved in the regulation of PNMT expression (Kvetnansky et al., 2009), we do not consider that increased *Hsd11b1* in adrenals of GF animals might substantially participate in up-regulation of *Pnmt*, because glucocorticoid-dependent

Pnmt and glucocorticoid-independent *Th* show similar expression pattern. Further studies will be necessary to understand the pathway how microbiota might affect the adrenal gland functions.

In summary, we demonstrated that the microbiota substantially affected behavior in social conflict and the expression profiles of genes associated with the peripheral metabolism of glucocorticoids and function and regulation of HPA and SAM axes. Our study expands on the previous works by showing for the first time in repeated stress, that the microbiota modulate the response of pituitary, adrenal gland and metabolism of glucocorticoids in peripheral tissues to repeated psychosocial stress.

5.2.2 The effects of microbiota on pituitary, adrenal gland and intestine exposed to acute restraint stress

Previous studies have shown that absence of microbiota is associated with exaggerated HPA axis response to psychological stressors and these changes are at least partly mediated by changes in brain neurochemistry (Clarke et al., 2013; Crumeyrolle-Arias et al., 2014; Sudo et al., 2004). In line with these observations, we showed (Vagnerová et al., 2019/Publication D) increased corticosterone response to acute restraint in GF mice and expanded this finding by demonstration that the microbiota affects also peripheral components of the HPA axis.

The acute restraint did not induce higher expression of *Pomc* in the pituitary, although we and others demonstrated the increase in pituitary *Pomc* expression after various chronic stress protocols (Aguilera et al., 1994; Vodička et al., 2018). This inconsistency could be due to distinct time points and stressors investigated, since POMC was upregulated after 15 min of restraint in rat pituitary (Ginsberg et al., 2006), but it returned to basal levels after 2 hours of restraint (Nemoto et al., 2013). On the other hand, downregulation of *Crhr1* gene expression during acute stress is in line with previous findings in rats (Nemoto et al., 2013; Rabadan-Dieh et al., 1996). Moreover, microbiota decreased the expression of *Pomc* and *Crhr1* in pituitary and had no effect on *Nr3c1* and *Fkbp5* expression. These result are inconsistent with our previous study, which did not report any effect of microbiota on expression of *Crhr1* and *Pomc* in pituitary, but showed effect of microbiota on *Fkbp5* expression (Vodička et al., 2018/Publication C). This discrepancy seems to be attributed to changes in treatment of the control group resulting from the nature of the experiments in isolator environment. In the first study (Vodička et al., 2018) control animals were group housed (4-5 per cage) and simultaneously transferred from the isolator through sterilized

transfer port. Although this procedure is stressful and it was done in the same way for both control and experimental group basal levels of *Fkbp5* might be activated by this unavoidable stress.

In order to maintain homeostasis, glucocorticoids act as a feedback inhibitors of hypothalamic CRH and pituitary POMC synthesis and secretion (Gagner and Drouin, 1985). Hence the final extent of glucocorticoid response depends on synthesis and secretion of CRH in hypothalamus, synthesis and secretion of ACTH in pituitary and responsiveness of adrenal gland to ACTH. Moreover, all these processes are modulated by negative glucocorticoid feedback. This feedback depends besides GR and its ligand also on the co-chaperon FKBP5 that decreases GR sensitivity to corticosterone and its nuclear translocation (Bekhbat et al., 2017). Since we didn't observe any effect of microbiota on *Nr3c1* and *Fkbp5* expression, we suggest that the microbiota does not influence the capacity of the negative feedback loop mediated by pituitary GRs in acute stress. On the other hand, the upregulation of pituitary *Pomc* and *Crhr1* expression in GF animals, might contribute to exaggerated HPA response to stress in these animals.

The production of glucocorticoids in adrenal cortex is mainly induced by ACTH and involves a cascade of enzymes that participate on the glucocorticoid biosynthesis. The expression of these enzymes needs to be effectively regulated and the nuclear steroidogenic factor 1 (SF1) stands as one of the key regulators of numerous steroidogenic enzymes in adrenal cortex (Miller and Auchus, 2011). Interestingly, even though the GF mice displayed higher reactivity of HPA axis, the expression of genes involved in ACTH signaling pathway in the adrenal gland such as *Mc2r*, *Sf-1*, and *Star* were not influenced by microbiota even if they were upregulated by restrain stress. Stress strongly upregulated the expression of *Sf-1* and *Star* and weakly *Cyp11a1*, without any effect on other steroidogenic enzymes. This finding corresponds with recent studies reporting lack of responsiveness of some genes encoding steroidogenic enzymes to acute restraint stress (Fallahsharoudi et al., 2015; Løtvedt et al., 2017). The rapid upregulation of *Star* and slight increase in *Cyp11a1* transcription are in line with current knowledge indicating the role of CYP11A1 in chronic maintenance of steroidogenesis (Miller and Auchus, 2011).

Interestingly, data of others (Sudo et al., 2004) and our unpublished data concerning acute immune challenge indicate, that hyper-reactivity of the HPA axis in GF mice is limited to psychological, but not systemic stressors. These two of stressors categories employ different

neuronal pathways and this aspect represent very interesting possibility in revealing the mechanisms behind microbiota regulation of the HPA axis.

5.3 Effect of stress and microbiota on extra-adrenal production of glucocorticoids

Both main pathways stimulated by stress, the HPA and SAM axes are potent modulators of immune functions depending on the nature, intensity and duration of stress (McEwen et al., 1997). Chronic stress can lead to immunosuppression and increased susceptibility to diseases (Cohen et al., 2012) or can enhance immune reactivity and induce glucocorticoid resistance, which prevents glucocorticoid-induced suppression of inflammation (Silverman and Sternberg 2012). As 11HSD1 locally alters the glucocorticoid availability, and thus may alter local immune responses, we also assessed the effect of various stressors on extra-adrenal corticosteroid production.

5.3.1 Effect of stress on local metabolism of glucocorticoid in lymphoid organs in rats

In stress hyper-reactive F344 rats, the expression of *Hsd11b1* was increased in spleen and mesenteric lymph nodes (MLN) by chronic psychosocial stress (Vodička et al., 2014/Publication A). In subsequent study (Ergang et al., 2018/Publication E), we analyzed the effect of psychosocial stress on glucocorticoid regeneration in lymphatic organs of stress hyper-reactive F344 and stress hypo-reactive LEW rats. The hypo-reactivity of the HPA axis in LEW rats is associated with vulnerability to immune diseases, whereas the F344 are inflammation resistant (Sternberg et al., 1992). We have shown that repeated social defeat increased the regeneration of corticosterone from 11-dehydrocorticosterone in the thymus, spleen and MLN. Considering the regulatory effects of glucocorticoids in immune cells (Ashwell et al., 2000; Mittelstadt et al., 2012; Tuckermann et al., 2007) and the expression of *Hsd11b1* in lymphocytes and immune organs (Ergang et al., 2011; Zhang et al., 2005), the stress-induced upregulation of corticosterone regeneration in lymphoid organs might represent a novel intracrine regulatory pathway in immune cells/organs. Similar stress-induced increase of 11HSD1 was recently demonstrated in liver (Corona-Pérez et al., 2015) and murine macrophages (Sesti-Costa et al., 2012). Compared with the F344 strain, LEW rats showed higher corticosterone regeneration in splenocytes of unstressed rats and in thymic and MLN mobile cells after stress but corticosterone regeneration in the stroma of all lymphoid organs was similar in both strains. The well-known augmented vulnerability of LEW rats to immune/inflammatory challenge (Sternberg et al., 1989) might be connected with a higher regeneration of corticosterone in thymocytes and MLN mobile cells of LEW rats exposed to stress. Considering that glucocorticoids can antagonize the signal transduction delivered through T cell

receptors in lymphocytes (Jondal et al., 2004; Mittelstadt et al., 2012), differences in corticosterone regeneration might distinctly modulate the activation and survival of T cells in the immune organs of both strains, however, more studies will be required to confirm this hypothesis. Comparable with our data, stressed LEW rats show decreased glucocorticoid receptor binding in immune tissues compared to F344 rats, even if there are no strain differences in the total glucocorticoid receptor levels in most immune tissues (Dhabhar et al., 1995). LEW rats also display higher plasmatic TNFα levels after immune stress induced by LPS challenge (Elenkov et al., 2008). Higher stress-induced plasma TNFα might contribute to increased glucocorticoid regeneration in LEW rats as cytokines participate in 11HSD1 regulation (Ergang et al., 2011, 2017/Publication F; Vodička et al., 2018/Publication C)

5.3.2 Effect of stress on glucocorticoid regeneration and cytokine milieu in murine mucosal immune system

Inflammation represents a potent stressor activating both HPA and SAM axes, which needs to be precisely regulated (McEwen et al., 1997). It was previously shown that colitis upregulates glucocorticoid regeneration in colon and MLN (Vagnerová et al., 2006). Here, we assessed the effect of experimental colitis on the expression of *Hsd11b1* in specific microanatomical compartments of the mucosal immune system (Ergang et al., 2017/Publication F). Colitis increased *Hsd11b1* expression in the colonic crypt epithelium (CCE), isolated intestinal lymphoid follicles (ILF), and MLN cortex, but not in the colonic lamina propria and the MLN paracortex and medulla. Colitis also upregulated the *Hsd11b1* expression in T cells of the spleen and MLN. Together, these data demonstrate that *Hsd11b1* expression is upregulated by inflammation both in the effector and inductive compartments of the colonic lymphoid tissue and in the secondary lymphoid organs. Each of the analyzed microanatomical compartments contain different cell types. The follicular subdivisions of the ILF and MLN cortex contain mainly B cells and stromal cells, but also populations of other cell types such as dendritic cells, follicle-associated epithelium and subsets of T cells. These subsets include T follicular regulatory cells and T helper cells, (Buettner and Lochner, 2016; Yu and Vinuesa, 2010). Our data do not provide information which cell types are responsible for upregulation of *Hsd11b1* expression. However, a comparison of our data with other results suggests that *Hsd11b1* was not increased in stromal or dendritic cells. Although the resident stromal cells are important in shaping a unique microenvironment in the lymph nodes (Ahrend et al., 2008), the absence of the stimulatory effect in the paracortex and medulla is in conformity with

the assumption that glucocorticoid metabolism is not upregulated in stromal cells. Moreover, in was shown that 11HSD1 activity operates at maximal rate in murine dendritic cells and is unaffected by additional stimuli (Soulier et al., 2013). On the other hand, we observed increased expression of *Hsd11b1* in T cells in colitis and it was previously demonstrated that vitro activation of the splenic and lymph node T cells is associated with increased 11HSD1 activity (Zhang et al., 2005).

Colitis also increased the expression of *Tnfα, Il-1β, Il-4, Il-10* and *Il-21* in ILF and partially in the MLN cortex, with no effect on *Ifnγ* and *Tgfβ*. *Hsd11b1* expression positively correlated with *Tnfα* and less strongly with *Il-21, Il-1β*, and *Il-4*. These data suggest that TNFα is the pivotal factor for *Hsd11b1* upregulation even if the effects of other cytokines cannot be excluded. This conclusion is in agreement with the previously shown stimulatory effects of cytokines on *Hsd11b1* expression in various in vitro experiments. 11HSD1 expression was shown to be upregulated by Th2/Th17 but not Th1 cytokines in airway mucosa and smooth muscle (Hu et al., 2006; Jun et al., 2014), fibroblasts (Hardy et al., 2006) and monocytes (Thieringer et al., 2001). 11HSD1 was also increased by the pleiotropic cytokines TNFα and IL-1β in a large variety of cell cultures of various origin (Ergang et al., 2011; Staab and Maser, 2010).

Cytokine expression can also be altered by stress (Ait-Belgnaoui et al., 2012, Audet et al., 2011, Gibb et al., 2011) and by the microbiota (Steinberg et al., 2014). In chronic psychosocial stress paradigm (Vodička et al., 2018/Publication C), we demonstrated that the presence of gut microbiota upregulated and stress downregulated the expression of cytokines in the colon irrespective of whether the cytokine belongs to the Th1, Th2 or Th17 pathway (Vodička et al., 2018). Similar stress-dependent downregulation of cytokine expression was observed after acute restraint (Vagnerová et al., 2019/Publication D). The decreased colonic expression of cytokines in stressed animals, which is partially modulated by the microbiota, supports previously described stress-induced immune suppression (Reber et al., 2011). We found correlation between *Hsd11b1* and cytokine expression and thus we hypothesize that *Hsd11b1* expression in colon is modulated by cytokine milieu and that the actions of cytokines are more potent in GF mice than in SPF mice. This conclusion is in agreement with the significantly increased expression of colonic *Hsd11b1* in control unstressed GF mice than in their SPF counterpart. Moreover, we have previously shown that TNFα and IL-1β upregulated colonic *Hsd11b1* in vitro (Ergang et al., 2011) and that the

expression of *Hsd11b1* in vivo was positively correlated with TNFα in MLN (Ergang et al., 2017/Publication F) The downregulation of colonic *Hsd11b1* in stressed animals can be related to the decreased expression of colonic cytokines in socially defeated animals. Additionally, glucocorticoids that are secreted in response to stress and that suppress cytokine expression might, at least to some extent, contribute to this decrease.

5.3.3 **Regulation of local steroidogenesis by microbiota in colon**

As the role of microbiota in shaping systemic glucocorticoid response to psychological stressor is well established (Sudo et al., 2004, Clarke et al., 2013; Crumeyrolle-Arias et al., 2014), the possible involvement of microbiota in extra-adrenal modulation of glucocorticoid signal and steroidogenesis is not clear. Based on previous findings showing that acute inflammation stress increases the local de novo synthesis of corticosterone in the intestine by upregulation of steroidogenic enzymes Cyp11a1 and Cyp11b1 (Cima et al., 2004), we further investigated if acute restraint stress affects expression of enzymes encoding local steroidogenesis in intestine (Vagnerová et al., 2019/ Publication D). Detailed analysis of steroidogenic genes in the colon revealed that expression of several genes of steroidogenic cascade is modulated by stress and microbiota, notably *Lrh-1*, which encodes the liver receptor homolog-1 (LRH-1) protein, a transcriptional factor essential for intestinal glucocorticoid synthesis and homolog of adrenal transcription factor SF-1 (Mueller et al., 2006). However, both transcription factors reacted to acute restraint differently. Although the *Sf-1* transcript was upregulated in the adrenal glands, expression of colonic *Lrh-1* was downregulated by stress in both GF and SPF animals. In contrast to *Lrh-1*, the effect of stress and microbiota on *Cyp11a1* showed interaction; stress downregulated the expression of *Cyp11a1* but only in GF animals. These data do not fit with previous observations, which have demonstrated upregulation of the expression of *Lrh-1*, *Cyp11a1*, and *Cyp11b1* in the intestine by acute inflammatory stress (Cima et al., 2004). This discrepancy probably reflects different quality of stress. Further experiments will be needed to dissect the exact mechanisms underlying the differences between acute restraint stress and inflammatory stress on intestinal synthesis of glucocorticoids. However, our results indicate that acute restraint stress might influence intestinal steroidogenesis and that this effect is modulated by microbiota.

6 Conclusions

Stress is often presented as a risk factor for many diseases. However, the stress reaction is designed to deal with life threatening situation and the deleterious effects usually stems from the potency of stress-activated pathways. When the amount of stress is excessive or the reaction to stressor is deregulated, the harmful side-effects begin to manifest. Therefore, precise regulation of stress response and its components is crucial. Stress reactivity is modulated by several factors including genetic predisposition or the environment. This Thesis was aimed at dissecting the mechanisms involved in regulation of the HPA axis and local glucocorticoid metabolism. To achieve this goal, diverse stress categories and various animals differing in stress reactivity were used. The main conclusions are summarized in the following points:

1. We assessed the impact of short-term psychological and chronic psychosocial stressor on activation of the HPA axis and glucocorticoid metabolism. Both stressor paradigms upregulated expression of *Hsd11b1* in limbic brain areas involved in HPA axis regulation, but not in the HPA axis itself. We also observed strain-dependent differences in *Hsd11b1* between F344 and LEW rats. Together with minimal stress-induced changes in *Nr3c1,* a gene encoding GR, we assume that 11HSD1 plays an important role in local regulation of glucocorticoid concentration and represents an important modulator of the HPA axis in limbic brain areas. (Publications A and B)
2. We found that psychosocial stress increased *Hsd11b1* expression in MLN and 11HSD1 activity in thymus, spleen and MLN. The increase of glucocorticoid regeneration was greater in LEW than F344 rats. The *Hsd11b1* expression was also elevated by inflammation in specific microanatomical compartments of the murine gut immune system and its expression correlated with the expression of *Tnfα* as well as other cytokines. Collectively, these results suggest that the increase of glucocorticoids mediated by 11HSD1 dampens the immune response locally and prevents it from overshooting. (Publications A, E and F)
3. The absence of microbiota increased plasma corticosterone levels in acute stress. Expression of pituitary *Pomc* and *Crhr1* and some genes encoding steroidogenic enzymes in adrenals were also modulated by microbiota. In chronic stress we showed stress- and microbiota-induced changes in expression of pituitary *Fkbp5* and genes

encoding key adrenal enzymes for catecholamine synthesis TH and PNMT, which indicates that microbiota represent an important factor in shaping the stress response, but the specific mechanisms still remain to be elucidated. (Publications C and D)

4. Both acute restraint stress and microbiota modulated the expression of some steroidogenic genes in colon, especially *Nr5a2*, which encodes LRH-1, the crucial transcriptional regulator of intestinal steroidogenesis. In contrast, chronic psychosocial stress down-regulated the expression of *Hsd11b1* and dampens the expression of a panel of cytokines depending on the presence or absence of gut microbiota. These results highlight the effect of microbiota on local extra-adrenal steroidogenesis and glucocorticoid metabolism. (Publications C and D)
5. The GF mice showed less escape behavior during resident-intruder test compared to their SPF counterparts indicating less anxiety. Decreased anxiety-like behavior in GF mice was described previously, we were the first to demonstrate under the conditions of psychosocial stress. (Publication D)

Together the results highlight the role of 11HSD1 in central feedback regulation of HPA axis response, local restriction of immune system and the importance of microbiota in regulating not only the HPA response to stress but also behavior and local extra-adrenal glucocorticoid metabolism.

7 List of publications enclosed in full length and contributions of the author

Publication A: **Vodička M***, Ergang P*, Mikulecká A, Řeháková L, Klusoňová P, Makal J, Soták M, Musílková J, Zach P, Pácha J. Regulation of 11β-hydroxysteroid dehydrogenase type 1 and 7α-hydroxylase CYP7B1 during social stress. PLoS One. 2014; 9: e89421. IF=3.234 (2014)

MV – Identification of distinct brain areas and performance of Laser capture microdissection; contribution to performance of stress protocol, animal sacrifice and tissue harvesting, analysis and interpretation of the data and writing the manuscript.

Publication B: Ergang P*, **Vodička M***, Soták M, Klusoňová P, Behuliak M, Řeháková L, Zach P, Pácha J. Differential impact of stress on hypothalamic-pituitary-adrenal axis: gene expression changes in Lewis and Fisher rats. Psychoneuroendocrinology. 2015; 53:49-59. IF=5.44 (2015)

MV – Management and performance of the stress protocol, identification of distinct brain areas and performance of Laser capture microdissection; contribution to animal sacrifice and tissue harvesting, analysis and interpretation of the data and writing the manuscript.

Publication C: **Vodička M©**, Ergang P, Hrnčíř T, Mikulecká A, Kvapilová P, Vagnerová K, Šestáková B, Fajstová A, Hermanová P, Hudcovic T, Kozáková H, Pácha J. Microbiota affects the expression of genes involved in HPA axis regulation and local metabolism of glucocorticoids in chronic psychosocial stress. Brain Behav Immun. 2018; 73: 615-624. IF=6.170 (2018)

MV – Corresponding author, design of the study, planning and management of the experiments; contribution to performance of the stress protocol, corticosterone measurement, animal sacrifice and tissue harvesting, analysis and interpretation of the data and writing the manuscript.

Publication D: Vagnerová K, **Vodička M**, Hermanová P, Ergang P, Šrůtková D, Klusoňová P, Balounová K, Hudcovic T, Pácha J. Interactions Between Gut Microbiota and Acute Restraint Stress in Peripheral Structures of the Hypothalamic-Pituitary-Adrenal Axis and the Intestine of Male Mice. Front Immunol. 2019; 10: 2655. IF=5.085 (2019)

MV – Design of the study, planning and management of the experiments, performance of the stress protocol, contribution to corticosterone measurement, animal sacrifice and tissue harvesting, analysis and interpretation of the data and writing the manuscript.

Publication E: Ergang P, Mikulecká A, **Vodička M**, Vagnerová K, Mikšík I, Pácha J. Social defeat stimulates local glucocorticoid regeneration in lymphoid organs. Endocr Connect. 2018 Dec;7(12):1389-1396. IF=2.474 (2018)

MV - contributed to performance of stress procedures, ACTH measurements, animal sacrifice and tissue harvesting, analysis and interpretation of the data and writing the manuscript.

Publication F: Ergang P, **Vodička M**, Vagnerová K, Moravec M, Kvapilová P, Kment M, Pácha J. Inflammation regulates 11β-hydroxysteroid dehydrogenase type 1 differentially in specific compartments of the gut mucosal immune system. Steroids. 2017 Oct;126:66-73. IF = 2.647 (2017)

MV – Identification and performance of Laser capture microdissection in colon and lymphatic organs, contributed to planning and management of the experiment, collitis induction, immunohistochemistry, animal sacrifice and tissue harvesting, analysis and interpretation of the data and writing the manuscript.

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9 Publications in full length