Peripartum Hypothalmus-Pituitary-Adrenal axis plasticity and its prevention by high-fat diet intake: Focus on the adrenal gland

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

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Prologue

The peripartum period represents a time of substantial plasticity in a wide-variety of systems of the maternal organisms in order to ensure the well-being of both the mother and the offspring. For its primary role in the maintenance of body homeostasis, the maternal Hypothalamus-Pituitary-Adrenal (HPA) axis profoundly adapts and animal models have been extensively used to study such plasticity given that particularly the findings from rodent studies were demonstrated to be highly translational to humans. Through basic and human research, several peripartum changes in HPA axis–associated hormonal levels as well as alterations in brain and pituitary morphology and physiology have been already identified. However, to my knowledge, despite the importance of the adrenal glands for the overall HPA axis function as the site of glucocorticoids synthesis and secretion, glucocorticoids that are the main HPA axis peripheral effectors, direct evidence of peripartum adrenal plasticity is missing.

Emerging evidence indicates that interference with peripartum HPA axis plasticity participates in the aetiology of mood and anxiety disorders; among them postpartum depression (PPD) and anxiety. Indeed, while the peripartum period is generally associated with increased calmness and decreased stress responses, for a substantial subset of mothers this period represents a time of particular risk for the onset of psychiatric disorders.

Intriguingly, human and rodent studies demonstrated that dysregulation of the basal- and stress-induced neuroendocrine HPA axis peripartum (re)activity feature in multiple pathologies, among them PPD, anxiety and obesity. Specifically, the previously mentioned illnesses are often characterized by unbalanced CORT and adrenocorticotropic hormone (ACTH) basal and stress-induced levels, ACTH which is the main CORT

secretagogue, finding that strongly suggests adrenal-mediated mechanisms may play a role. However, to my knowledge, it is unknown if the adrenal glands adapt during the peripartum period, and if so, whether disruption of such changes may contribute to the aetiology of peripartum-associated pathologies.

In the following Chapter 1, I will first provide an overview of the stress response physiology with a detailed description of adrenal gland's morphology, steroidogenic pathways and cholesterol delivery, as the main part of my thesis will focus on these aspects. Furthermore, I will describe the hormonal adaptations occurring across the estrous cycle and the peripartum period particularly regarding ACTH and CORT levels. The last part of the general introduction chapter provides a description of the animal models that have been shown to induce dysregulation of the maternal HPA axis that are, therefore, commonly used to study potential mechanisms underlying PPD, anxiety and the mechanisms of comorbidity between obesity and these disorders.

The following three experimental chapters describe and discuss in detail the experiments I performed whereas in Chapter 5 a general discussion of the present thesis findings is provided, together with future perspectives and conclusions.

Chapter 1: General Introduction

1.1. The stress response

There are two major components that participate to mount an appropriate physiological response upon stress exposure, the HPA axis and the sympathetic nervous system. The former is involved in the regulation of several physiological functions, e.g. energy metabolism and immune function (Dallman et al., 1995, Straub et al., 2001, Ulrich-Lai and Herman, 2009) and its major role is to maintain body homeostasis and thus, concert the physiological response to external or internal changes or to threat exposure.

After exposure to a stressor, sensory information is driven to the brain to recruit the specific effectors to mount an appropriate response. Specifically, HPA axis activity is driven by corticotrophin-releasing factor (CRF) whereas its co-secretagogue vasopressin (AVP) is believed to amplify the stress response (Gillies et al., 1982); both CRF and AVP are released from the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus at the level of the median eminence. They reach anterior pituitary corticotropic cells through the hypophyseal portal system where they stimulate ACTH release and production mainly inducing calcium influx. ACTH is the main stimulator of adrenal steroidogenesis and reaches the adrenal glands *via* the circulatory system (Young and Akil, 1985, Familari et al., 1989, Aguilera and Rabadan-Diehl, 2000). The CRF-containing neuronal population of the PVN receives a great variety of projections, among them ascending brainstem fibers that promote HPA axis activation, angiotensinergic projections from the circumventricular organs that activate the HPA axis in case of osmotic stress and from several basal forebrain and hypothalamic pathways (Gillies et al., 1982). The main HPA axis peripheral effectors are glucocorticoids (cortisol in humans

and corticosterone in rodents; hereafter CORT), steroid hormones produced and secreted from the adrenal gland cortex in an intermittent manner (pulsatile and circadian) and acutely after stress exposure (Gan and Quinton, 2010) (details about adrenal morphology and steroidogenic pathways that are relevant for my thesis are provided in the introduction Section 1.2).

CORT binds two receptor subtypes that are widely distributed throughout the body, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The former regulates several functions such as body mineral balance, while the latter participates to the regulation of a multitude of physiological processes, among them immune and cardiovascular function, reproduction and cognition and energy metabolism, particularly regarding glucose (Oakley and Cidlowski, 2013). MR and GR belong to the nuclear hormone receptors superfamily and are located in the cytosol when inactive although plasma membrane located receptors have also been identified; the membrane-located receptors are believed to mediate glucocorticoids effects that do not rely on genomic mechanisms (Zhang et al., 2012). As glucocorticoids are lypophilic they can freely cross the membrane barrier to bind to the receptor (MR or GR), which becomes activated and translocates into the nucleus where it modulates the activity of target promoter genes (Bamberger et al., 1996). Besides their peripheral effects, glucocorticoids exert several function at the brain and pituitary levels, effects that directly regulate HPA axis function. Indeed, their action is of primary importance in the maintenance of basal HPA activity and its stress-induced activation and for the termination of the stress response *via* feedback mechanisms (De Kloet et al., 1998) (Figure (Fig.) 1).

In contrast to the HPA axis-related stress response activation, which takes several minutes, the sympathetic nervous system responds within seconds to challenges with its activity coordinated by the locus coeruleus and its effector being adrenaline released from the adrenal medulla (Chrousos and Gold, 1998) (Fig. 1). The locus coeruleus is the main

source of noradrenaline in the brain; it receives CRF projections from the central nucleus of the amygdala, the bed nucleus of the stria terminalis and from the PVN that directly modulate its activity and elicit its stress-induced activation, projections that terminate in the peri-coerulear region where locus coeruleus dendrites extend (Valentino and Van Bockstaele, 2008). The locus coeruleus projects to preganglionic sympathetic neurons, which are predominantly cholinergic, in the intermediolateral cell column of the thoracolumbar spinal cord. These preganglionic neurons project directly to the adrenal chromaffin cells or to prevertebral and paravertebral ganglia where they synapse with noradrenergic postganglionic neurons that, in turn, project to the target organs. Activation of the sympathetic nervous system is necessary to cope with stressful situations or threats and results in an increase of the circulating levels of adrenaline, mainly from adrenal chromaffin cells production, and noradrenaline, from sympathetic neuron's terminal. Through interactions with the two main adrenoreceptor classes, namely alpha- and beta-adrenoreceptors, adrenaline and noradrenaline coordinate the cardiovascular, respiratory, gastrointestinal, renal and endocrine systems function and increase energy mobilization (Molinoff, 1984).

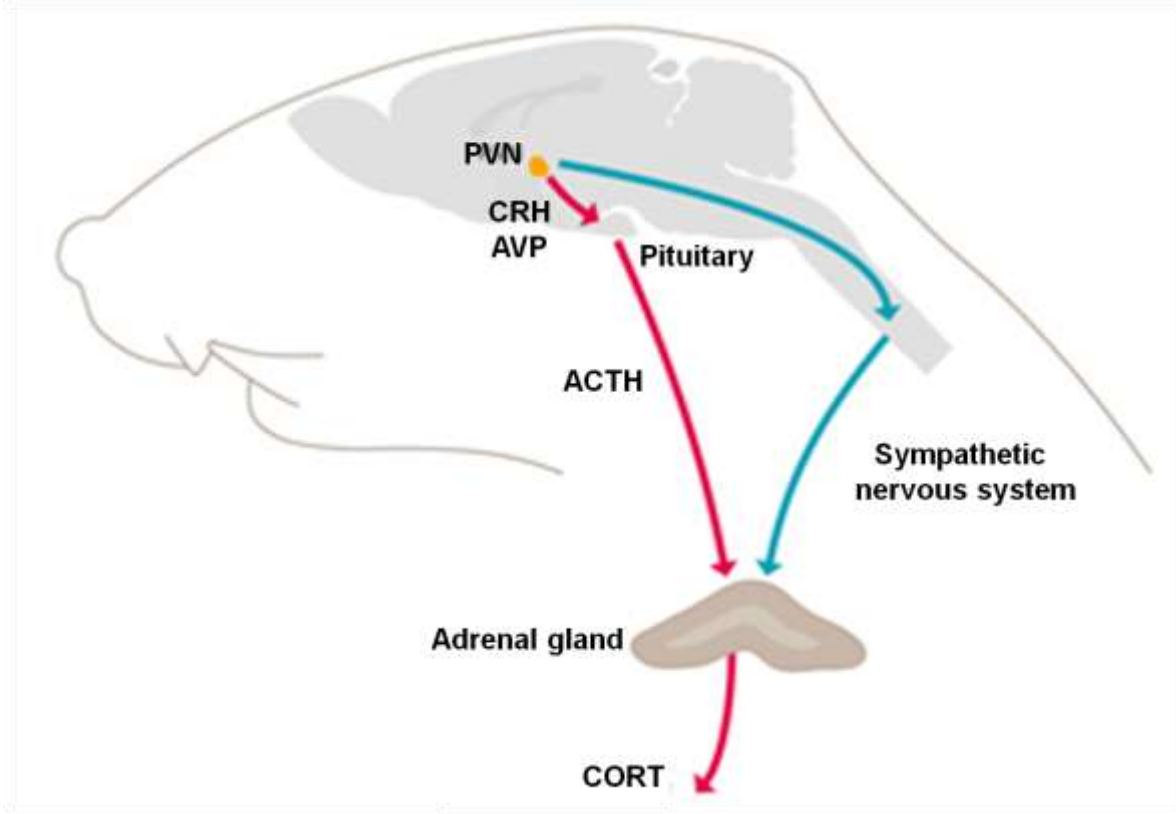


Figure 1. Schematic representation of the Hypothalamus-Pituitary-Adrenal (HPA) axis and sympathetic nervous system physiological response to stress

The sympathetic nervous system and the HPA axis orchestrate the stress response. The sympathetic pregnanglionic neurons from the toracolumbar spinal cord are activated upon stress exposure. These neurons innervate directly the adrenal medulla. HPA axis activation upon stress exposure results in corticotrophin releasing factor (CRF) and vasopressin (AVP) release from the paraventricular nucleus of the hypothalamus (PVN). CRH and AVP stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, ACTH that induces glucocorticoids (CORT) synthesis and release form the adrenal cortex. Adapted from www.kalsbeekgroup.nl/.

The experimental sections of the present thesis focus on studies that have been performed in rats and mice particularly at the adrenal gland level. Therefore, in the following introduction chapters, I will specifically describe some aspects of rodent physiology with a special focus on the adrenal glands and HPA axis-related hormones. However, for completeness, cross references to the human physiology, particularly when

markedly divergent to the rodent condition are reported; although less details are available for humans.

1.2. The adrenal glands

The adrenal glands are paired endocrine glands located in the retroperitoneal space close to the kidneys. The glands are macroscopically divided into two main regions, which are structurally and functionally distinct, the inner medulla and the outer cortex, and are surrounded by a connective tissue envelop termed the capsule (Fig. 2). Since the rat adrenal glands comprise the main body of my thesis, below I will describe the physiology, structure and function of these glands in detail.

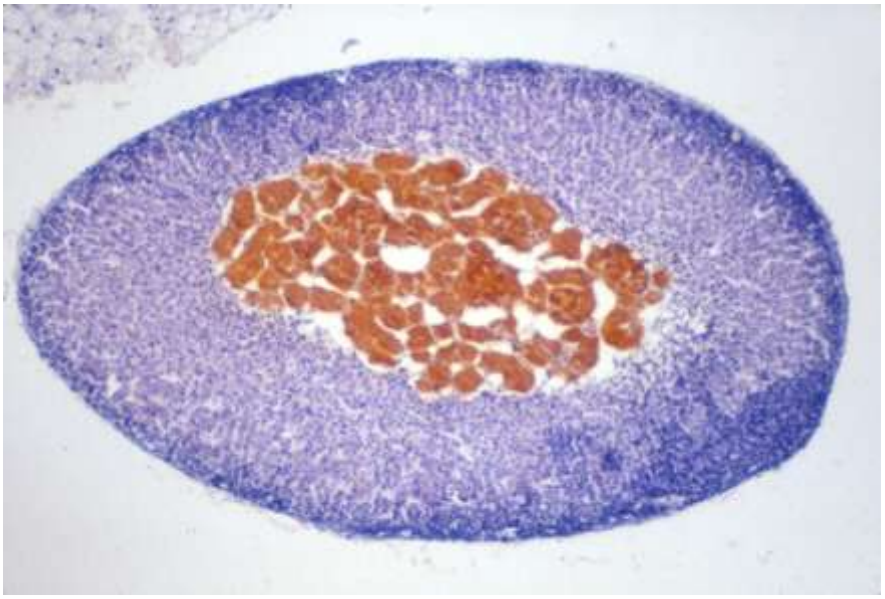


Figure 2. Macroscopic structure of the rodent adrenal glands: the adrenal medulla and cortex

The chromaffin medullary cells constitute the adrenal glands core; in this photograph from a rat adrenal, the medulla is yellow coloured due to chromium staining. The area surrounding the medulla is the adrenal cortex. From www.pathbase.net

1.2.1. The adrenal cortex

As previously mentioned the cortex represents the adrenal structure located directly below the capsule and is dedicated to steroid hormone synthesis. Adrenal steroids are divided in two classes: glucocorticoids, with carbohydrate metabolism-regulating action and mineralocorticoids, with electrolyte balance-regulating functions. The adrenal cortex can be divided in three main regions depending on the hormonal production: the outer glomerulosa, the intermediate located fasciculata and the inner reticularis zone (Fig. 3). The main product of the glomerulosa zone is aldosterone, a mineralocorticoid hormone that enhances renal sodium and water retention whereas the fasciculata and reticularis areas are responsible for CORT production (Rosol et al., 2001). In humans, the reticularis produces also androgens, specifically dihydroepiandosterone and androstenedione, hormones that in rodents are produced mainly by the gonads (Rodgers, 1990, Sharpe, 1990).

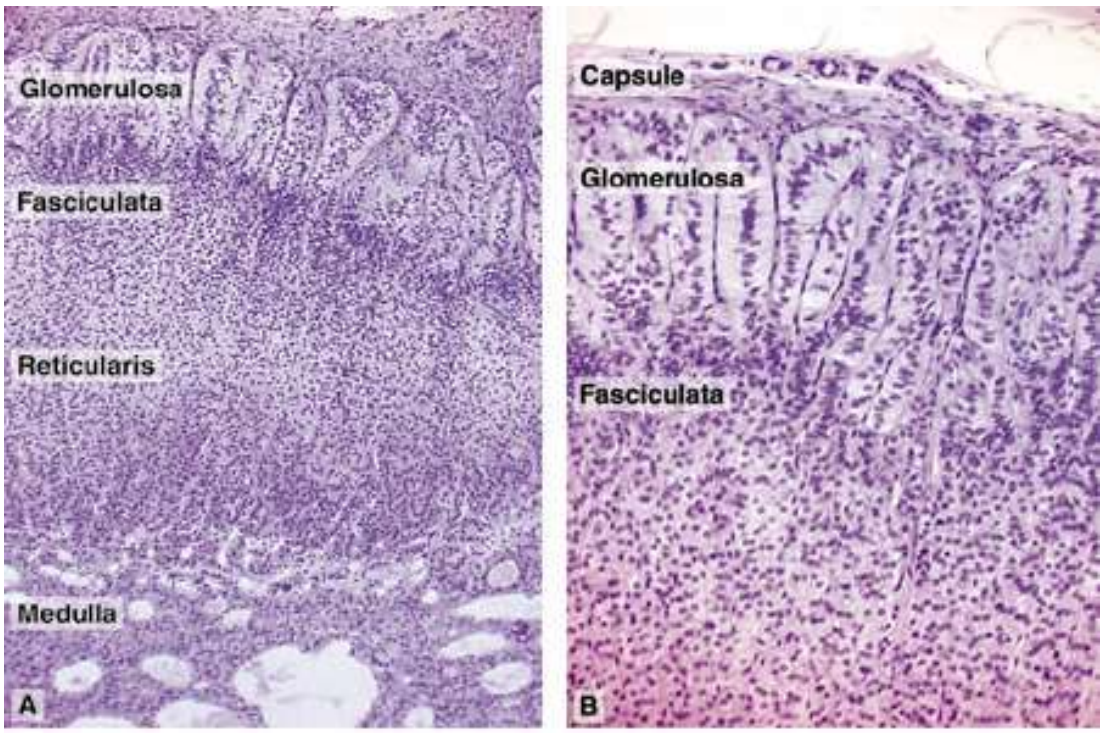


Figure 3. Photomicrographs of hematoxylin and eosin stained sections from the rat adrenal

The adrenal medulla is surrounded by the innermost adrenal cortex area, the reticularis which is followed by the intermediate adrenal cortex area, the fasciculata (A). The outermost zone of the adrenal cortex is the glomerulosa (A) that is surrounded by the capsule (B). Adapted from Junqueira and Carneiro, Histologie, Springer, 2005.

1.2.1.1. CORT synthesis

As mentioned above, CORT is produced in the adrenal fasciculata and reticularis zones through a multiple-step reaction that is mainly controlled by ACTH. Optimal steroidogenesis is achieved upon ACTH binding to the adrenal ACTH receptor (ACTH-R), a seven transmembrane protein G coupled receptor whose activation increases cyclic adenosine monophosphate (cAMP) intracellular levels *via* adenylate cyclise activation that, in turn, activates protein kinase (PK) A (Gallo-Payet and Payet, 2003). Activation of the cAMP-PKA pathway is involved in both, the acute and chronic regulation of steroidogenesis and cholesterol is the precursor of all steroid hormones. The acute response occurs within minutes upon stimulation and induces cholesterol mobilization to the inner mitochondria membrane where the enzyme, cytochrome P450 (CYP) side-chain cleavage enzyme (CYP11A1 or P450scc), catalyzing the first steroidogenic reaction is located (see below). The chronic response to cAMP-PKA activation involves transcription of genes encoding for components of the steroidogenic machinery (Hu et al., 2010). The first step of adrenal steroidogenesis is, therefore, the transport of unesterified cholesterol to the inner mitochondrial membrane, transport that involves the steroidogenic acute regulatory (StAR) protein (Stocco, 2000). The two major classes of enzymes responsible for CORT biosynthesis are the cytochrome P450 (CYP) and the hydroxysteroid dehydrogenase (HSD) enzymes. Specifically, the P450 enzymes are membrane bound proteins associated with the mitochondrial membrane or the endoplasmic reticulum and

catalyze the hydroxylation and cleavage of the steroid substrates (Payne and Hales, 2004). On the other hand, HSD enzymes are involved in the reduction and oxidation of steroids and are membrane-bound enzymes located in the endoplasmic reticulum and in the mitochondria (Payne and Hales, 2004).

The first step in steroid hormone synthesis is catalyzed by the enzyme CYP11A1 that cleaves the carbon side-chain of the cholesterol molecule to produce pregnelonone (Fan and Papadopoulos, 2013) (Fig. 4). Pregnenolone is converted by the enzyme 3β-HSD into progesterone that, in turn, is converted in 11-deoxycorticosterone through the action of CYP21A2, enzyme located in all three adrenal cortex regions. The final step in CORT biosynthesis is mediated by the enzyme CYP11B1, which converts deoxycorticosterone in CORT (Mukai et al., 1993). In the glomerulosa zone, CORT is converted in aldosterone through the action of the enzyme aldosterone synthase (CYP11B2).

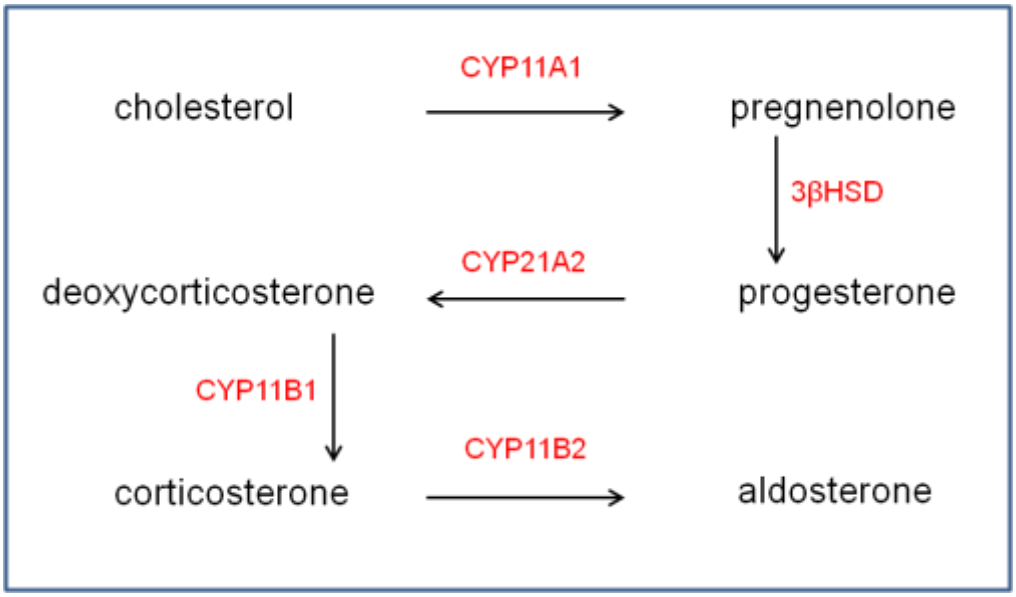


Figure 4. Schematic representation of the steroidogenic process in the rodent adrenal

The rate-limiting step in CORT biosynthesis is the conversion of free cholesterol into pregnenolone through the action of the cytocrome P 11A1 (CYP11A1; P450scc) enzyme. 3β hydroxysteroid dehydrogenase (3βHSD) converts pregnenolone in progesterone that is, in turn, converted in deoxycorticosterone by CYP21A2. The final step in CORT production is catalyzed by the enzyme

CYP11B1 whereas, in the adrenal glomerulosa, CORT is converted to aldosterone by the enzyme CYP11B2.

The differential distribution of the steroidogenic enzymes confers the specificity of the hormonal production to each adrenal cortex area. Indeed, the enzyme CYP11B2 is exclusively located in the adrenal glomerulosa (Vinson, 2003). On the other hand, the steroidogenic enzyme expression pattern is species specific; this variability underlies the diversity in adrenal hormonal production across species. Thus, in rodents, the adrenal fasciculata and reticularis do not produce cortisol and androgens, which are the main hormonal products of the human adrenal gland. Indeed, mice and rats adrenals do not express the enzyme CYP17, which is involved in the conversion of pregnenolone and progesterone into cortisol and dehydroepiandrostenedione (Perkins and Payne, 1988, Rainey et al., 2002).

1.2.2. Adrenal cholesterol utilisation

As stated above, cholesterol is the substrate for steroid hormone synthesis and its availability for conversion into pregnenolone by the enzyme CYP11A1 represents the rate limiting step in steroidogenesis. Therefore, besides the importance of ACTH to stimulate CORT synthesis, cholesterol supply is also critical for optimal hormonal production (Hu et al., 2010).

The adrenal gland's cholesterol is supplied from several sources (Fig. 5): it can be uptaken through receptor-mediated endocytosis *via* low density lipoprotein receptor (LDLR) or selectively uptaken from LDL and high density lipoproteins (HDL); a process

mediated by the scavenger receptor class B type 1 (SRB1). Cholesterol can be *de novo* synthesised within the gland from acetyl CoA with being the rate limiting enzyme and, finally, adrenal cholesterol esters stores can be recruited (Kraemer, 2007, Hu et al., 2010).

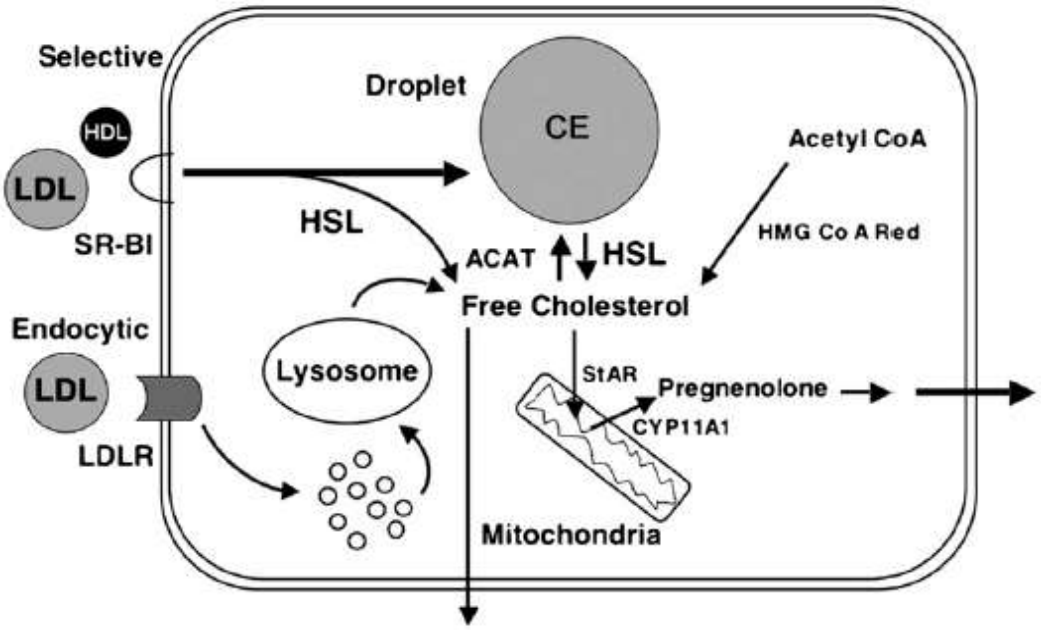


Figure 5. Adrenal cholesterol delivery and storage dynamic

Cholesterol can be derived from three different sources. It can be selectively uptaken from plasma high density lipoproteins (HDL) and low density lipoproteins (LDL) through the action of scavenger receptor B1 (SRB1) whereas LDL cholesterol can also be recruited *via* LDLR internalization mediated by the LDL receptor (R). Cholesterol esters (CE), stored in adrenal lipid droplets and synthesised from free cholesterol by the enzyme acyl Coa cholesterol acyltransferase (ACAT), can be hydrolyzed by the hormone sensitive lipase (HSL) enzyme. Cholesterol can be *de novo* synthesised from Acetyl Co through a multiple-step reaction involving hydroxymethylglutaryl CoA reductase (HMG Co A Red) activity. Free cholesterol is transported to the inner mitochondrial membrane by steroidogenic acute regulatory (StAR) protein and the first step in steroidogenesis is catalyzed by the enzyme cytocrome P(CYP) 45011A1 that converts cholesterol into pregnenolone (Kraemer, 2007).

Cholesterol is transported through the aqueous circulatory system packed in plasma lipoproteins, which are complex aggregates of lipids and proteins. Four lipoprotein classes can be identified based on the density of the lipoproteins aggregates that form upon ultracentrifugation, chilomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL), which differ in both composition and physical properties. The high steroidogenic activity of the adrenal cortex results in pronounced cholesterol metabolism and lipid exchange with the circulation. The adrenals express two lipoprotein receptors that uptake cholesterol via two distinct mechanisms, the LDLR and the SRB1, which are discussed in detail in the two next sections.

1.2.2.1. Receptor mediated cholesterol endocytosis

The LDLR, localized at the plasma membrane, interacts exclusively with LDL - and cholesterol uptake through this pathway is named receptor-mediated uptake. LDL directly interacts with the LDLR, and this complex migrates to coated pits, which are plasma membrane regions specialized in internalization of substances from the circulation. The coated pits first invaginate, and then come away from the plasma membrane as vesicles that fuse with early endosomes in the cytoplasm where the complex LDL-LDLR gets degraded because of the endosomal pH fall (Fielding and Fielding, 1996, Hu et al., 2010). Here, the LDLR must undergo enough conformational change to release the ligand without getting denatured in the acidic endosomal environment. After dissociation, the LDLR leaves the endosome, apparently *via* incorporation into a membrane of a vesicle that buds from the endosome surface. These receptor-mediated endocytotic mechanisms allow reuse of the receptor every 10-20 minutes and an LDLR can make up to 150 trips through the endosomes without losing its function (Goldstein and Brown, 1985, Brown and Goldstein, 1986). The released (or internalised) LDL cholesterol esters are hydrolized in

the early endosomes and free cholesterol accumulates in the late endosomes/lysosomes. Free cholesterol can then be esterified through the action of the enzyme acyl CoA: cholesterol acyltransferase (ACAT) for storing in cytoplasmic lipid droplets or used immediately. There have been two ACAT subtypes identified to date, ACAT1 and ACAT2, but in adrenal cells ACAT1 is the major isoenzyme and contributes to more than 90% of the total ACAT activity (Chang et al., 2006). In addition to its storage into lipid droplets, cholesterol delivered via this LDLR-mediated pathway may be directed to the plasma membrane or to the mitochondria for steroid hormones synthesis (Sugii et al., 2003, Miller, 2007).

1.2.2.2. Cholesterol selective uptake

The SRB1 is responsible for "selective cellular cholesterol uptake". This receptor is a ~ 82 kilodalton cell surface glycoprotein, which is highly expressed in tissues that are dependent on cholesterol from plasma lipoproteins for hormone production like the adrenal glands. Selective uptake via the SRB1 differs from the previously described receptor-mediated endocytosis as it occurs on the cell surface with cholesterol esters being internalized without lipoprotein endocytosis. Moreover, whilst the LDLR interacts exclusively with LDL, SRB1 binds a broader variety of ligands, among them HDL and native-, oxidized- and acetylated-LDL. SRB1 cholesterol ester uptake occurs in two steps: binding of the lipoprotein to the receptor and transfer of the lipids from the lipoprotein to the plasma membrane. SRB1 was suggested to bind plasma lipoproteins *via* mechanisms involving direct protein-to-protein contact. Specifically, an amphiphathic α-elices motif present on different lipoproteins is hypothesised to be involved (Williams et al., 2000). The mechanisms involved in cholesterol ester transfer to the plasma membrane are poorly understood to date. One hypothesis is that the SRB1 forms a sort

of “channel” through which the cholesterol esters penetrate the cells driven by a concentration gradient (Vinals et al., 2003). Cholesterol esters, after being internalized in the adrenal cell, are hydrolyzed by a neutral cholesterol ester hydrolase (Sparrow and Pittman, 1990), which is believed to be hormone sensitive lipase (HSL) in adrenal cells (Kraemer and Shen, 2002). Existing literature reports that in murine adrenals and ovaries the fatty acids component of the cellular cholesterol esters differs from the circulating one; strengthening the hypothesis that after uptake from plasma lipoproteins, cholesterol esters are hydrolyzed and then re-esterified before storage (Egwim and Kummerow, 1972). Free cholesterol from this selective uptake pathway may be driven to the mitochondria for steroidogenesis and in an unstimulated cell it would probably be stored in lipid droplets (details about adrenal lipid droplets dynamic are reported in the following Section) (Connelly and Williams, 2003).

1.2.2.3. Adrenal lipid droplets

Cellular lipid droplets are considered to be a mobile, dynamic and complex organelle with a neutral lipid core surrounded by a monolayer of phospholipids and proteins (Martin and Parton, 2005). The primary function of lipid droplets is the storage and supply of cholesterol and fatty acids. In adrenal cells and other steroidogenic cells, the droplets are particularly enriched in cholesterol esters to provide the precursor for steroid hormone production. Lipid droplets form in the endoplasmic reticulum *via* mechanisms, which have been just partially addressed (Fig 6). It is for example still obscure whether they remain in physical contact with the endoplasmic reticulum after their formation or whether become totally independent organelles (Martin and Parton, 2006).

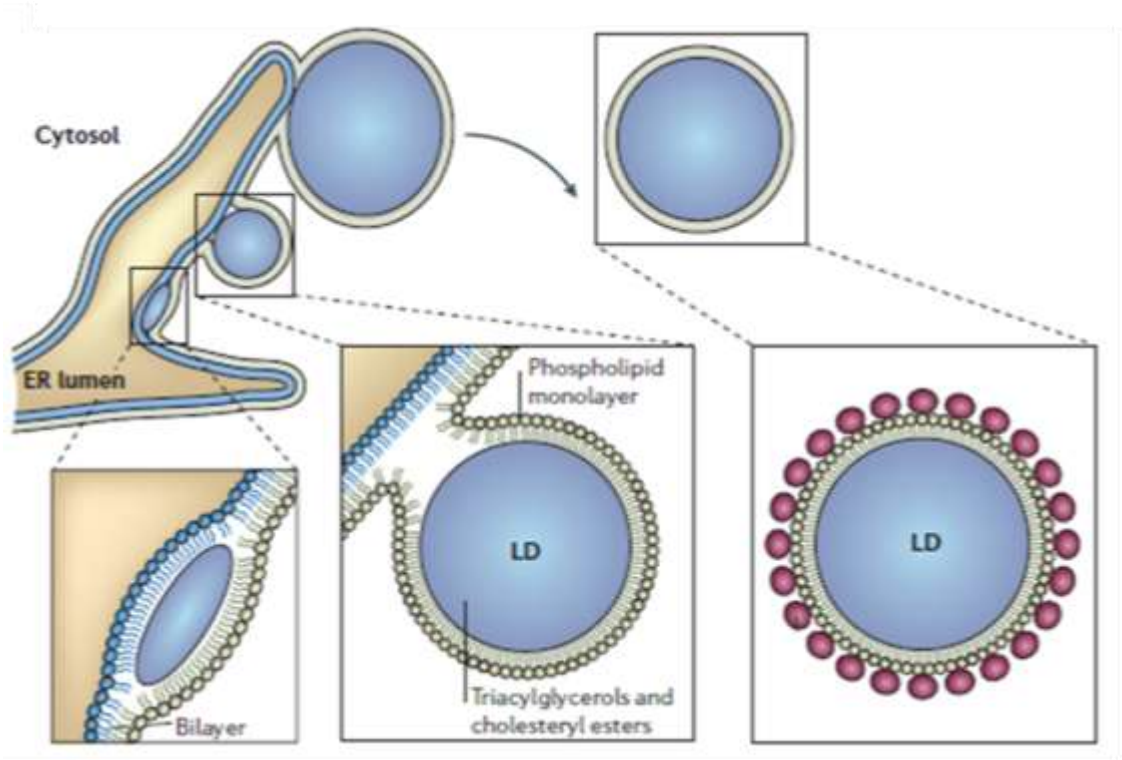


Figure 6. Model for cellular lipid droplet formation

Mature lipid droplets (LD) are hypothesised to bud from the endoplasmic reticulum (ER) to form mature organelle with a triacylglycerols and cholesterol esters core surrounded by a phospholipid monolayer. Adapted from (Martin and Parton, 2006).

Lipid droplets are rapidly formed and catabolized depending on the cell requirements for cholesterol and lipids. It is, therefore, intuitive how ACTH, the main adrenal tropic hormone, strongly affects adrenal lipid droplets physiology. For example, long term ACTH administration was shown to induce the depletion of glomerusa zone droplets, with parallel increase in plasma aldosterone (Mazzocchi et al., 1986). Interestingly, several acute and chronic stress paradigms were demonstrated to impact adrenal lipid droplets in animal models. Specifically, 60 minutes of high temperature exposure was reported to markedly increase plasma ACTH and CORT, together with depletion of adrenal fasciculata zone droplets in rats (Koko et al., 2004). The effect of chronic stress on the

pool of cholesterol esters within the adrenal appears to depend on the stress paradigm and species employed. In a study performed in rats subjected to chronic noise exposure, stress reduced adrenal fasciculata-associated lipid droplets (Oliveira et al., 2009), while chronic psychosocial stress in mice does not affect this parameter (Fuchsl et al., 2013). Animal models of chronic stress are important to study the pathogenesis of diseases linked to HPA axis dysregulation like depression or anxiety (Slattery et al., 2012). Therefore, understanding of possible adrenal participation in the pathology of these diseases is particularly relevant.

1.2.2.4. Adrenal cholesterol synthesis

The adrenal glands can also synthesise cholesterol from acetyl Co A *via* a multistep reaction. The enzyme catalyzing the rate-limiting step of the cholesterol production is HMGCR, a reductase that converts hydroxymethylglutaryl-Co A into mevalonate. HMGCR is an important regulatory loci of cholesterol synthesis and both, sterol and non-sterol agents, were shown to impact on HMGCR function and expression. Cultured cells contain a maximal amount of HMGCR when incubated in the absence of serum. Addition of LDL to the culture suppresses HMGCR expression by 90% (Goldstein and Brown, 1977, Faust et al., 1982) suggesting that cholesterol uptake inhibits intracellular cholesterol *de novo* synthesis. Mevalonate is not exclusively a precursor for cholesterol synthesis. Several other non-sterol substances synthesised via mechanisms involving HMGCR may therefore, regulate HMGCR together with lipoproteins derived cholesterol (Brown and Goldstein, 1980).

Adrenal synthesis may supply adequate cholesterol for steroidogenesis. However, it is likely that plasma lipoproteins are recruited when steroidogenesis is sustained like during

the peripartum period, especially when considering that the maternal organism shows hyperlipidemia with plasma lipoprotein cholesterol increase that parallels the rise in CORT (Desoye et al., 1987).

1.2.3. The adrenal medulla

The adrenal medulla is located in the organ's core and represents about 10% of the total gland size. This tissue is considered as a paraganglia because sympathetic pre-ganglionic neurons of the splanchnic nerve innervate the main medulla cell type, namely the chromaffin cells, *via* acetylcholine release. This neurotransmitter is synthesised through a multi-step reaction with the enzyme tyrosine hydroxylase (TH) catalysing the rate limiting step in catecholamines synthesis (Fernstrom and Fernstrom, 2007). TH is regulated acutely by phosphorylation at different sites whose phpsphorylation increase TH activity (Haycock and Wakade, 1992) chronically by protein synthesis. Adrenal TH mRNA, protein levels and phosphorylation are partially regulated by neurotransmitters released by the splanchnic nerve and, therefore, variation of the expression level are believed to mirror changes in the sympathetic firing to the adrenals (Brooks et al., 1997, Ong et al., 2011). Acetylcholine is hydrolyzed with consequent firing interruption through the action of the acetylcholinesterase enzyme (Fig. 7). Specifically, sympathetic pre-ganglionic neurons originating from the intermediolateralcell column of the spinal cord, enter the adrenal capsule and, after branching and travelling throughout the cortex forming an extensive neuronal network, innervate the adrenal medulla (Strack et al., 1989, Parker et al., 1993, Mravec, 2005). Normally, sympathertic pre-ganglionic neurons synapse in the ganglia with acetylcholine being the neurotransmitter involved, whereas the originating postganglionic neurons reach and innervate target organs *via* noradrenaline release. Chromaffin cells, which release adrenaline or noradrenaline upon

stimulation, are therefore, considered as modified post-ganglionic sympathetic neurons. Postganglionic sympathetic fibers, mainly noradrenergic, have also been identified in the adrenal medulla and cortex, processes that have been found to be generally anatomically associated with blood vessels (Toth et al., 1997, Delarue et al., 2001).

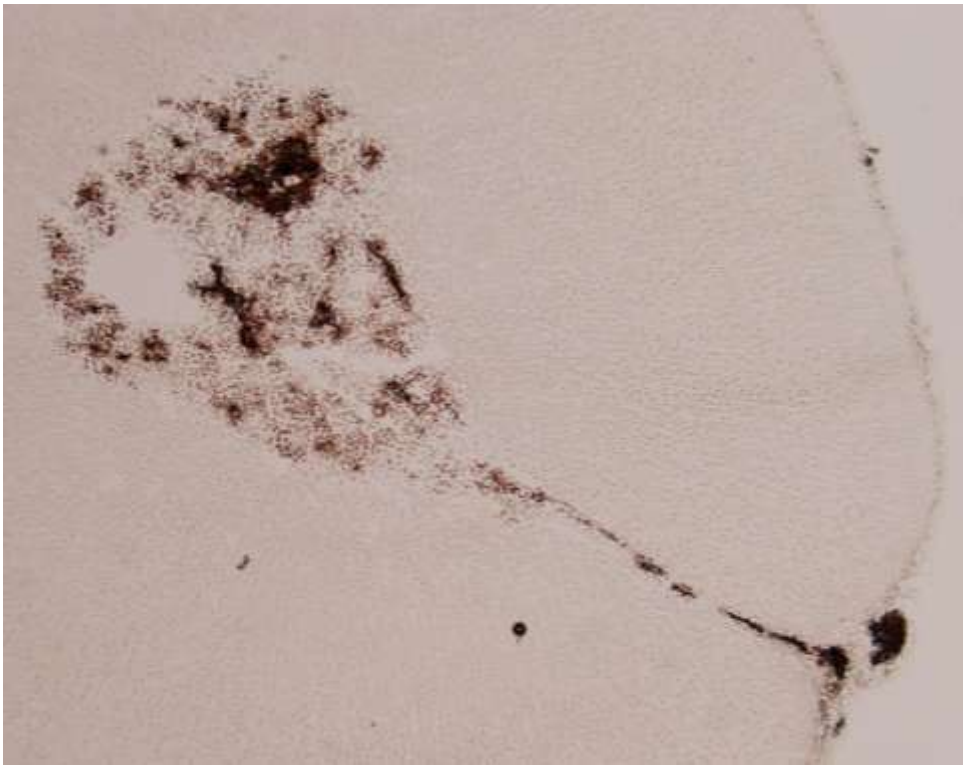


Figure 7. Rat adrenal glands acetylcholinesterase enzyme localization

The enzyme acethylcholinesterase localization can be visualized *via* the histochemical thiocholine method (Schober et al., 1997). The brown-coloured material results from acetylcholinesterase activity after adrenal slice exposure to the acetylcholinesterase substrate acetylcholine iodide.

Two sub-types of chromaffin cells can be distinguished depending on their secretory adrenergic or noradrenergic activity. In the adrenal medulla of the adult rat, 80-85% of the cells are adrenergic and primarily located adjacent to cortical cells and the remainder are therefore noradrenergic and mainly located in the centre of the medulla (Coupland, 1965).

Adrenaline and noradrenaline are stored in cytoplasmic secretory granules and released in response to a physiological signal by exocytotic mechanisms (Diaz-Flores et al., 2008). Adrenergic and noradrenergic chromaffin cells are innervated by different sympathetic neurons that are functionally divided. This differential innervation of the two adrenal chromaffin cell populations is believed to underlie the selective release of adrenaline or noradrenaline in response to different insults. Thus, for example, insulin-induced hypoglicemia has been shown to activate adrenaline-secreting cells, whereas acute cold exposure has been reported to induce mainly noradrenaline release in the rat (Vollmer et al., 1992, Morrison and Cao, 2000). Brain areas that receive somatic and visceral information from the periphery including caudal raphe nuclei, ventromedial medulla, rostral ventrolateral medulla, A5 cell group, and PVN regulate the activity of the sympathetic neurons innervating the adrenal medulla (Mravec, 2005). Neurons whose cell body is located primarily in the adrenal medulla that innervate the adrenal cortex have also been identified. Specifically, vasoactive intestinal peptide-, substance P- and neuropeptide Y-containing ganglion cells have been recognised and they constitute the intrinsic adrenal innervation network (Engeland, 1998, Mravec, 2005). The rodent adrenal content of the above mentioned neuropeptide has been shown to vary depending, for example, on the age of the animal or after food deprivation (Chua et al., 1991). However, the physiological meaning and the mechanisms underlying these changes are still unclear (Bornstein and Ehrhart-Bornstein, 2000).

Steroidogenic and chromaffin cells have been to shown to be in physical contact (Fig. 8) and evidence of a mutual influence on the secretory activity of these different cells types has been reported by both *in vitro* and *in vivo* studies. For example, bovine adrenocortical cells basal CORT release *in vitro* was reported to be 10-fold higher in the presence of chromaffin cells; a coculture that also resulted in an increased expression of mRNA for several steroidogenic enzymes (Haidan et al., 1998). Adrenaline-mediated mechanisms

are hypothesised to be involved since stimulation of bovine cortical cells with adrenaline was reported to influence CORT secretion and accumulation of the mRNA encoding for the enzyme cholesterol side-chain cleavage cytochrome P450 (Ehrhart-Bornstein et al., 1991), the enzyme that catalyzes the first step of steroid hormones production (see Section 1.2.1.1). Moreover, ablation of the gene encoding for TH, the key enzyme involved in the synthesis of catecholamine, is associated with reduced basal CORT levels *in vivo;* despite no changes in plasma ACTH (Bornstein et al., 2000). On the other hand, adrenal steroids have also been demonstrated to influence the expression of enzymes involved in catecholamine synthesis (Haase et al., 2011). However, the exact mechanisms underlying the adrenal cortex and medulla cross-talk are poorly understood (Schinner and Bornstein, 2005).

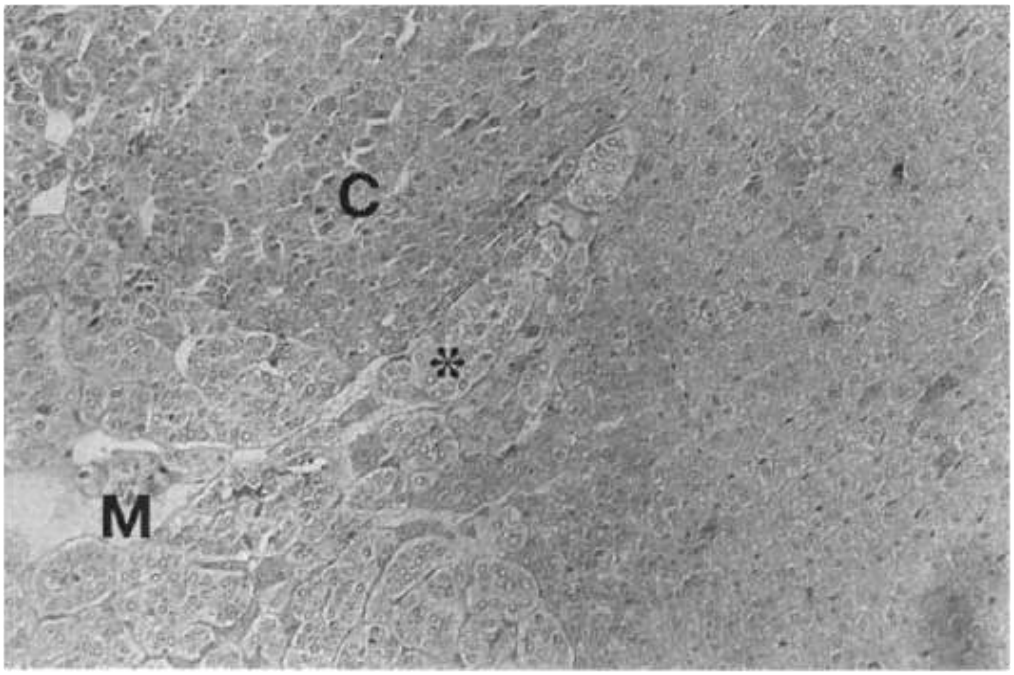


Figure 8. Adrenal cortical-chromaffin physical contact

Light micrograph of the rat adrenal gland stained with hematoxylin-eosin; C cortex, M medulla, * medullary ray in the cortex (Pignatelli et al., 1998).

1.3. HPA axis and hormonal changes across the estrous cycle

1.3.1. The rat estrous cycle

In rodents, the estrous cycle, the ovarian cycle occurring in non-pregnant adult females, takes between three to five days resulting in multiple ovulations, while in women it takes between 25 and 30 days (called menstrual cycle) and only one oocyte is ovulated (Silberstein and Merriam, 2000, Westwood, 2008). The main hormones that are involved in the regulation of the estrous cycle-associated events are hypothalamic gonadotropin-releasing hormone (GnRH), pituitary luteinizing- and follicle-stimulating hormones (LH and FSH, respectively), progesterone and estrogens.

In the rat, female sexual maturity is reached immediately after the vaginal orifice opens around postnatal day 30, which is also when hormonal cycling begins. Hypothalamic GnRH neurons, mainly localised in the mediobasal hypothalamus, release GnRH in a pulsatile manner to the medial eminence, from where GnRH reaches the pituitary *via* the portal blood system, where it stimulates the synthesis and secretion of LH and FSH (Charlton, 2008). The estrous cycle is characterized by four alternating phases named diestrous, proestrous, estrous and metestrous (Westwood, 2008). During diestrous, the longest estrous cycle phase (50 to 55 hours), the reproductive tract prepares for the potential oocyte implantation. Usually, in the afternoon of the following 12 to 14 hours of the proestrous phase, LH and FSH levels that are otherwise low across the other estrous cycle phases, increase (Marcondes et al., 2001). These proestrous-associated hormonal changes are necessary to trigger ovulation that occurs during the 25 to 27 hours estrous phase. Specifically, ovulation takes place when prolonged and transitory exposure of the pituitary gland to high estrogens levels triggers the LH peak that, in turn, stimulates follicle rupture (Perez and Apfelbaum, 1992). During estrous, the female is receptive to the male

and, therefore, copulation may occur. After follicle rupture, the follicle differentiates into corpus luteum through a process named luteinisation. The corpus luteum is defined as a transient endocrine gland important for progesterone production (Stocco et al., 2007). Progesterone, whose production is sustained by LH, is necessary for the uterus changes to allow the potential embryonic implantation. When conception does not take place, the corpus luteum loses its function and is converted into the corpus albicans; with the subsequent progesterone loss allowing a new cycle to begin (Stocco et al., 2007) (Fig. 9). The estrous phase is followed by metestrous (6 to 8 hours), when estrous-associated changes in the reproductive tract subside in the absence of conception. In the morning of metesterous, a rise in estrogens synthesis from follicle production induces a decrease in FSH levels that results in follicle selection (Chaffin and Vandevoort, 2013). Progesterone levels increase during metesterous and diestrous and decline afterwards; progesterone levels peak a second time again at the end of the proestrous phase (Marcondes et al., 2001).

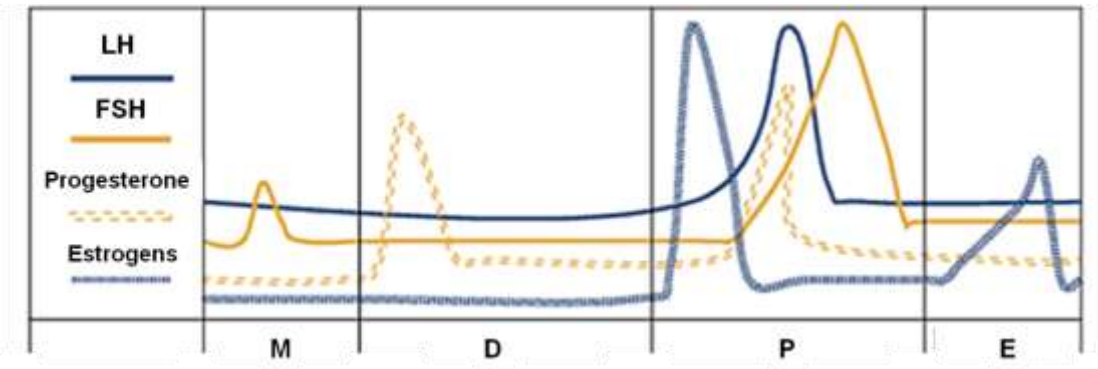


Figure 9. LH, FSH, progesterone and estrogens fluctuation across the estrous cycle

The estrous cycle is characterized by four phases: metestrous (M), diestrous (D), proestrous (P) and estrous (E). At proestrous, a peak in estrogens, LH and FSH is observed. Specifically, LH surge triggers ovulation and sustains progesterone proestrous-associated peak. After ovulation, the hormones return to baseline. Another progesterone rise is observed at diestrous whereas an estrogens peak is necessary to decrease FSH levels, events that result in follicle selection. Adapted from (Emanuele et al., 2002)

Together with progesterone, the hormone prolactin (PRL) participates to induce corpus luteum involution. PRL is a polypeptide hormone secreted from various organs and tissues including the anterior pituitary, the brain and the mammary gland. Its release, particularly from the adenohypophyisis, follows an estrous cycle-dependent pattern characterized by a surge at proestrous that is necessary for luteolysis. PRL exerts a variety of important functions that allow reproduction and body homeostasis maintenance by participating to the immune response, osmoregulation and angiogenesis (Freeman et al., 2000). PRL exerts its effects through binding to numerous isoforms of the PRL receptor (PRL-R), a single membrane-bound protein (Freeman et al., 2000). These isoforms differ in the intracellular domain length and sequence whereas the extracellular structure is identical. A short, intermediate and long receptor form have been described in the rat and there is evidence that suggests that the different isoforms have independent biological activity (Binart et al., 2010). PRL-Rs are localized in several brain regions and peripheral tissues, among them the pituitary and adrenal and mammary glands, heart, uterus and ovary (Nagano and Kelly, 1994). Sex hormones, as well as oxytocin (OXT) and dopamine, are known to influence the P-associated PRL surge (Freeman et al., 2000). For example, administration of an antiserum to estradiol in the morning of diestrous has been shown to block the PRL surge (Neill, 1972), while a single estradiol injection was shown to increase plasma PRL following a time frame similar to the proestrous-associated PRL peak (Neill et al., 1971). Administration of a specific antiserum for OXT prior to the PRL surge was reported to reduce the systemic elevation in PRL concentrations in the afternoon of proestrous (Sarkar, 1988).

1.3.2. The human menstural cycle

In women, the menstrual cycle is divided in three phases: the proliferative (follicular) phase, ovulation (second phase) and the secretory (luteal) phase. The events that precede ovulation and the activities that result in follicle rupture are similar to the above described estrous cycle-associated events with the important exception that rodents are capable of multiple ovulations whereas women ovulate exclusively one oocyte per cycle. On the other hand, the events associated with the maintenance of an active corpus luteum drastically diverge (Chaffin and Vandevoort, 2013). Specifically, in women, in the absence of conception a long functional luteal phase (from seven to twelve days) develops during which the corpus luteum actively engages in steroidogenic function and expresses inhibin A whose main function is to suppress FSH secretion preventing follicle growth. In rodents, a proper secretory phase is missing and, therefore, new follicle maturation takes place less than 24 hours after the previous ovulation (Chaffin and Vandevoort, 2013).

1.3.3. Plasma ACTH and CORT levels across the rat estrous cycle

Basal and stress-induced HPA axis (re)activity in terms of plasma ACTH and CORT levels have been shown to vary across the estrous cycle (Viau and Meaney, 1991, Atkinson and Waddell, 1997, Figueiredo et al., 2002). Sex hormone fluctuations were hypothesised to play a role taken that sex steroids have been demonstrated to influence adrenal steroidogenesis (Viau and Meaney, 1991, Nowak et al., 1995, Figueiredo et al., 2007). Interestingly, plasma ACTH and CORT have been shown to disconnect across the estrous cycle under basal conditions (Atkinson and Waddell, 1997) and also after acute stress exposure (Viau and Meaney, 1991) suggesting that adrenal-mediated mechanisms might participate to the estrous cycle associated changes in HPA axis (re)activity. Specifically, in an elegant and detailed study from 1997, Atkinson and colleagues

monitored the circadian release of ACTH and CORT across the estrous cycle in Wistar rats. An extra group of males was added to include sex comparison in the study. Circadian dependent rhythmicity in plasma CORT was observed in both males and females independently from the estrous cycle phase; the degree of the rhythm was also unaffected by sex and the estrous cycle. On the other hand, the absolute CORT plasma levels markedly differed across the groups (Fig. 10). Specifically, average daily plasma CORT concentrations were higher at proestrous compared to all the other groups; the lowest concentrations were detected at estrous and in the following metestrous and diestrous phases the levels rose but remained significantly lower than the proestrous-associated average daily plasma CORT concentrations. Males CORT levels did not differ from the concentrations observed in females at estrous but were lower compared to those detected at metestrous, diestrous and proestrous; findings that have been recapitulated also by another study performed in Sprague Dawley rats (Figueiredo et al., 2002). The differences in the daily CORT averages observed were attributed to differences in the peak values since CORT trough-associated levels did not differ across the groups. Interestingly, whilst ACTH levels were reported to display a circadian rhythm with highest values detected before lights off, neither the sex nor the estrous cycle affected ACTH levels (Fig. 10).

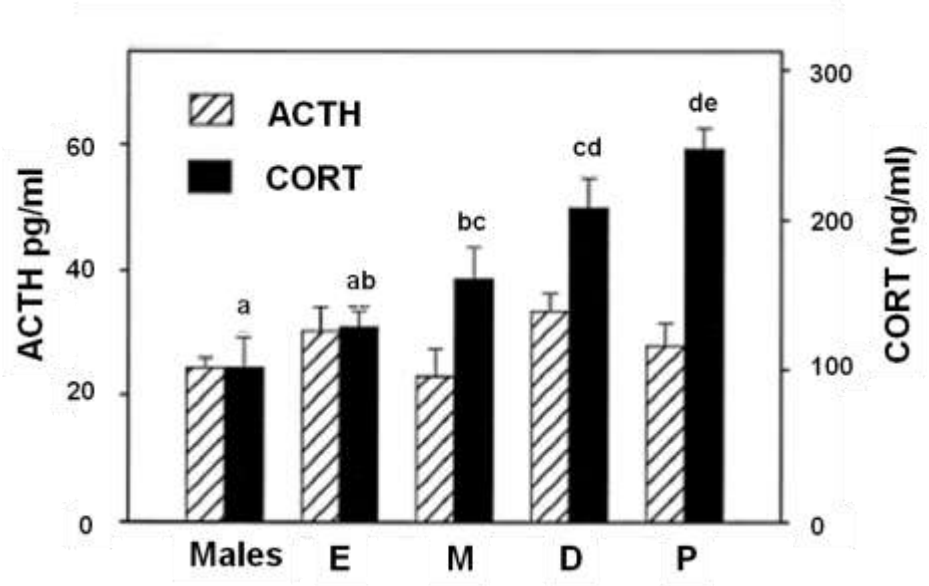


Figure 10. ACTH and CORT daily average levels in male rats and across the estrous cycle

Average ACTH (striped columns) and CORT (full columns) daily levels are represented (n=five/nine per group). Hormonal levels measured in males and females at estrous (E), metestrous (M), diestrous (D) and proestrous (P) are represented. Groups without common notations differ significantly. Adapted from (Atkinson and Waddell, 1997).

Stress-induced ACTH and CORT levels have also been shown to vary across the estrous cycle (Viau and Meaney, 1991). Specifically, although 20 minutes restraint stress has been reported to increase ACTH and CORT levels in Long Evans rats at diestrous, proestrous and estrous, ACTH levels in the proestrous group were doubled compared to both diestrous and estrous at the end of the stress procedure. Moreover, plasma CORT was increased after stress termination and higher at proestrous compared to diestrous and estrous females. In contrast, the CORT differences observed across the groups were less pronounced compared to the marked diversity observed in the ACTH stress-induced levels. Estrous cycle associated changes in adrenal gland sensitivity to ACTH and sex hormones fluctuation have long been hypothesised to account for the previously mentioned disconnection between plasma ACTH and CORT. In line with this, estrogens through binding to the estrogens R α and β (Green et al., 1986, Nilsson and Gustafsson, 2011), have been shown to impact on adrenal steroidogenesis. Specifically, estradiol was reported to enhance basal, but not ACTH-induced, CORT secretion from isolated adrenocortical cells *in vitro* (Nowak et al., 1995). In contrast, an *in vivo* study from Figueiredo and colleagues suggested that estrogens potentiated adrenocortical response to stress in female rats with a mechanism hypothesised to involve the adrenal glands. In more detail, estradiol injection in ovariectomized females increased CORT secretion; despite reduced PVN activation measured *via c-fos* mRNA activation and plasma ACTH levels after acute restraint stress exposure (Figueiredo et al., 2007). This discrepancy between *in vitro* and *in vivo* findings may be related to that fact that estrogens may affect also the synthesis of steroids hormones precursor, cholesterol. Specifically, the promoter

of the rat HMGCR, key enzyme for cholesterol synthesis, contains a tissue specific estrogen-responsive region (Di Croce et al., 1999).

LH has also been shown to influence adrenal steroidogenesis with LH overexpressing female mice displaying an 80% increase in adrenal size and 14-fold increase in serum CORT levels (Kero et al., 2000). Moreover, *in vitro* incubation of adrenal cells resulted in higher CORT production from LH overexpressing cells than controls, suggesting that adrenal mechanism may adapt to sustain the previously mentioned elevated CORT production *in vivo* (Kero et al., 2000). LH estrous cycle-associated fluctuations might, therefore, participate to the ACTH and CORT dissociation observed in females. Brain-mediated mechanisms may play a role as well in this imbalance. Choi and colleagues showed that lesion of the anteroventral region of the bed nucleus of the stria terminalis in male rats prevented the expected plasma ACTH peak after stress exposure without affecting CORT increase. However, it remains to be determined whether a similar response would be observed in female rats. Taken together, these results suggest that brain and adrenal mediated changes may bypass pituitary ACTH to stimulate CORT production (Choi et al., 2007).

1.4. Hormonal and behavioural changes across the peripartum period

1.4.1. Pregnancy and lactation

In all mammalian species, the peripartum period is associated with profound physiological, emotional and behavioural changes that act in concert to ensure the health and well-being of both, the mother and offspring. These changes include substantial alterations in the HPA- and hypothalamus-pituitary-ovary axes. In most mammalian species pregnancy-

associated estradiol and progesterone release follow the same pattern as in rodents (Rosenblatt et al., 1988). Specifically, estrogen and progesterone progressively increase during pregnancy to levels 50- and 10-fold higher than observed in virgins, respectively, and rapidly reverse to their pre-pregnancy levels after parturition. These levels drop within a few days of parturition, remain low during the first half of lactation (approximately 10 days in rodents and until a monthly cycle resumes in women - which can take up to 180 days) and begin to increase when follicular maturation starts again (Bridges, 1984, Brunton and Russell, 2010). In contrast, the progesterone profile differs between women and rodents, as it remains low throughout lactation in women but increases to levels observed in late pregnancy by the third day of lactation in rats (Grota and Eik-Nes, 1967, Taya and Greenwald, 1982, Rosenblatt et al., 1988, McNeilly, 2001). In the rat, the ovary produces hormones through pregnancy while in other species, including humans, fetoplacental production is more important (Taya and Greenwald, 1982). Specifically, the corpus luteum becomes in the rat a substantial site for progesterone production. This is a specific feature of the corpus luteum because the follicle indeed primarily produces estrogens. A constant cholesterol supply is needed to sustain progesterone synthesis throughout pregnancy with plasma lipoproteins being the primary source (see Section 1.2.2 for more details on cholesterol delivery for steroidogenesis). As previously mentioned, progesterone levels increase stating at pregnancy day (PD) 5 and they gradually rise to peak around PD 15-16 before rapidly decreasing around PD 19 (Pepe and Rothchild, 1973). Progesterone stimulates its own secretion and it protects corpus luteum cells from death (Telleria and Deis, 1994). Locally-produced estradiol stimulates progesterone production and promotes, particularly at mid-pregnancy, the vascularisation of the corpus luteum (Tamura and Greenwald, 1987).

1.4.1.1. Prolactin and Oxytocin

PRL exerts several important tasks during pregnancy and some of them at the corpus luteum level where PRL-R are expressed. Specifically, the longer PRL-R isoform is thought to mediate the effect of PRL on the luteal cells whereas the short isoform stimulates angiogenesis in the endothelial cells (Freeman et al., 2000). Importantly, LH induces an up-regulation of PRL-R during luteinisation and PRL-R-knockout (KO) mice display lack of implantation and consequent sterility (Grosdemouge et al., 2003). Pituitary lactotrophs, which synthesise PRL, represent the 20-50% of the pituitary cells depending on the sex and on the reproductive status of the animal. In the pituitary of neonatal rats another cell population called mammosomatotrophs secrets PRL. These cells differentiate into lactotrophs in the presence of estrogens and of a maternal signal that reaches the pups at early lactation throughthe milk (Porter et al., 1991, Freeman et al., 2000). Lactotrophs store a large amount of PRL that can be released *via* a calcium-dependent exocytotic mechanism. At early pregnancy, PRL is secreted following a circadian pattern characterized by two daily surges that cease after mid-pregnancy when pituitary PRL production stops and the decidua layer of the uterine lining takes over this task until the trophoblast begin the production of PRL-like hormones (Grattan and Averill, 1990, Prigent-Tessier et al., 1999). PRL levels remain low until they increase at late pregnancy before labour (Grattan and Averill, 1990). During lactation, the suckling physical stimulus induces PRL release and this release pattern is superimposed to the circadian rhythm. However, suckling-induced plasma PRL levels are increase when nursing occur at the time of the circadian peak (Freeman et al., 2000).

Together with the PRL system, OXT circuitry is highly activated in the peripartum period because of its involvement in the regulation of several pregnancy- and lactation-associated physiological processes. Blockade of these two systems was shown to

negatively interfere with phenomena like labour and lactation. Thus, blockade of OXT central signalling *via* central infusion of OXT selective antagonist at late gestation was reported to delay the suckling-induced systemic OXT release and to affect pups and dams weight (Lipschitz et al., 2003) while PRL and PRL-R KO mice failed to produce milk (Horseman et al., 1997, Ormandy et al., 1997). Involvement of brain OXT and PRL systems for the display of maternal behaviour is also known. Specifically, PRL-R KO mice (Lucas et al., 1998) and rats with reduced brain PRL-R expression induced by antisense infusion (Torner et al., 2002) show impaired maternal behaviour, while intracerbroventricular infusion of OXT antagonist reduces the display of arched back nursing between lactation day 2 and 5 (Bosch and Neumann, 2008) (see Section 1.4.4 for PRL and OXT involvement for the maternal stress hyporesponsiveness).

1.4.2. Changes in HPA axis function across the peripartum period

Maternal HPA axis function shows profound plasticity across the peripartum period with both basal and stress-induced ACTH and CORT plasma levels being profoundly affected. Thus, basal CORT levels have been shown to be increased from mid-pregnancy despite minor changes in plasma ACTH whereas a marked reduction in the hormonal response elicited by acute stress exposure has been documented in several species including humans (Carr et al., 1981, Allolio et al., 1990, Douglas et al., 1998, Shanks et al., 1999, Brunton and Russell, 2003, Douglas et al., 2003, Windle et al., 2013).

Pregnant and lactating women have been shown to be less stress reactive compare to non-pregnant subjects with breastfeeding women appearing to be even less stress reacting when compared to non breastfeeding mothers (Rosenblatt et al., 1994, Heinrichs et al., 2001, Glynn et al., 2004, Heinrichs and Koob, 2004). Here, I go into detail of just

one example in which saliva CORT secretion was measured before and after 60 seconds (s) immersion of the dominant hand in ice cold water in women towards the end of pregnancy and eight weeks post-partum. Whilst non-pregnant women from the control group react to the stress with a significant increase in salivary CORT, neither pregnant nor lactating women showed differences in CORT release (Kammerer et al., 2002). Rodent studies reported that CORT secretion after exposure to 20 or 30 minutes restraint stress (da Costa et al., 1996, Johnstone et al., 2000), five minutes elevated plus maze exposure and 60 or 90 s forced swim (Neumann et al., 1998) was reduced in pregnant rats compared to virgins. Data collected from mice studies follow the same trend. Douglas and colleagues showed that pregnant mice were less responsive to novel environment exposure or forced swim in term of CORT secretion compared to virgins (Douglas et al., 2003). Evidence of reduced HPA axis responsiveness during lactation has also been reported. Specifically, lactating animals on LD 3 and 4 and between LD 10 and 14 displayed lower CORT levels compared to virgin animals when killed after 30 minutes restraint stress (da Costa et al., 1996). Lactating Sprague-Dawley rats did not show any change in ACTH and CORT plasma levels after noise stress exposure, whereas this stress procedure elicited an increase in both hormones in virgins (Windle et al., 1997). Hillerer and colleagues observed reduced CORT response in dams on LD 8 compared to virgin rats after 60 s forced swim exposure. Interestingly, ACTH secretion did not differ among the groups pointing out that other mechanisms beyond changes in ACTH release might mediate the hypo-response in terms of CORT secretion, mechanisms that might involve adrenal glands plasticity (Hillerer et al., 2011). Peripartum changes in HPA axis stress-induced reactivity have been hypothesised to be mediated, at least in part, via alterations in the CRF system. Decreased CRF mRNA within the PVN in late pregnancy (Johnstone et al., 2000) and lactation (Fischer et al., 1995), decreased median eminence CRF content in late pregnancy (Ma et al., 2005), together with reduced pituitary CRF receptor binding from mid-pregnancy (Neumann et al., 1998), reflect changes that have

been speculated to contribute to maternal stress hyporesponsiveness. Moreover, evidence for reduced excitatory firing to the PVN have been reported in studies performed with pregnant and lactating rats (for review see (Slattery and Neumann, 2008)). Thus, indication of a decrease in both basal and stress-induced noradrenaline release in the PVN at late pregnancy have been documented (Douglas et al., 2005) with the reduction of the noradrenergic signalling continuing into mid-lactation (Douglas, 2005) in the rat.

Besides the previously mentioned changes in ACTH and CORT levels after stress exposure, evidence of altered basal HPA axis activity has also been repeatedly shown across pregnancy and lactation. Specifically, a detailed analysis of plasma ACTH and CORT across pregnancy in the rat reported that basal CORT plasma levels were increase in pregnant rats compared to virgins at diestrous from mid-pregnancy, difference that reached the maximal extent when virgins were compared to animals at pregnancy day 22. Both morning and evening plasma CORT levels were affected whilst the amplitude of the circadian CORT rhythm did not vary. These changes were not accompanied by marked variation in plasma ACTH (Atkinson and Waddell, 1995) (Fig. 11).

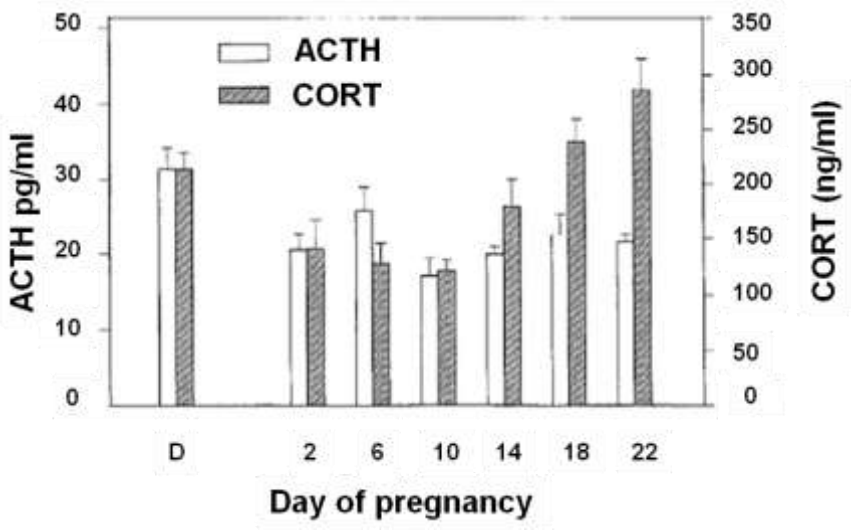


Figure 11. Basal plasma ACTH and CORT levels across pregnancy

Average ACTH (white columns) and CORT (gray columns) daily levels are represented (n=five/nine per group). Each bar shows the mean for each group. Adapted from (Atkinson and Waddell, 1997).

An increase in the basal CORT levels have been observed also across lactation with the circulating basal CORT levels starting to decline around LD 15-18, probably as a consequence of reduced suckling from the pups around this time when the ingestion of solid food begins (Voogt et al., 1969). Specifically, Fischer and colleagues reported that CORT levels were increased on LD 1, 3, 7 and 19 compared to diestrous virgins; plasma CORT returned to diestrous levels one day after weaning. Interestingly, plasma ACTH was reduced in lactating dams compared to virgins at all time points (Fischer et al., 1995). However, whilst basal high CORT levels across lactation has been extensively reported (Walker et al., 1992, Hillerer et al., 2011), findings on plasma ACTH are more controversial. Indeed, besides the previously mentioned decrease in plasma ACTH observed by Fischer and colleagues (Fischer et al., 1995), others reported no changes (Atkinson and Waddell, 1995, Hillerer et al., 2011) or even increased basal plasma ACTH in lactating animals compared to virgins (Walker et al., 1992, Shanks et al., 1999). These discrepancies might derive from several factors including differences in the strain of the animal or blood sampling time points and conditions.

Peripartum-associated plasticity of the AVP system has been hypothesised to participate in the maintenance of high basal HPA axis activity in the lactating rat. Thus, increased neuronal AVP expression within the PVN (Walker et al., 2001), together with an enhanced pituitary sensitivity to AVP (Toufexis et al., 1999), were theorized to induce, at least in part, high basal CORT production.

1.4.3. Adrenal plasticity might sustain the dissociation of plasma ACTH and CORT peripartum

Whereas central and pituitary adaptations of the CRF and AVP systems described above, together with the profound peripartum-associated plasticity of the OXT and PRL networks, have been shown to account for some of the changes in HPA axis peripartum activity, the discrepancy between ACTH and CORT suggests that the adrenal gland may also adapt during this period. However, although this has often been hypothesised, to my knowledge, direct evidence is still missing.

Indeed, while central adaptations might explain the peripartum-associated changes in plasma ACTH, they do not explain the previously mentioned discrepancy of plasma ACTH and CORT levels. It has long been hypothesised that these dissociation might rely on altered adrenal sensitivity to ACTH across the peripartum period; although this hypothesis has never been actually examined. Additionally, when considering the peripartum-associated basal hyperCORT, this explanation is controversial as it would require a switch from high sensitivity of the adrenal ACTH-R at basal condition, to a lower sensitivity to allow stress hyporesponsiveness. Considered the overall “peculiar” peipartum HPA axis functionality, it is, therefore, dubious to theorize that the higher basal adrenal sensitivity to ACTH may suddenly decrease to dampen CORT production after stress exposure. It is more probable that mechanisms beyond adrenal sensitivity to ACTH are involved.

1.4.4. Consequences of peripartum-associated changes on behaviour

The previously mentioned changes in HPA axis peripartum (re)activity are hypothesised to link to the maternal behavioural phenotype characterized by increased calmness and decreased anxiety-related behaviour but also to be necessary for the barrage of behaviours that mothers display in order to feed, protect, warm and take care of the litter (Fleming and Rosenblatt, 1974). Lactating females also perform differently when tested for aggressive-related behaviours and spatial memory. Indeed, rat mothers when exposed to unfamiliar intruders in the home cage during the maternal defence test are more aggressive than nulliparus females (Neumann et al., 2001, Bosch and Neumann, 2012). The increase in spatial memory of pregnant and lactating rats was shown *via* Morris water maze testing where pregnant females travelled shorter distances and displayed shorter latencies to escape than non-pregnant rats (Galea et al., 2000). Several studies reported that anxiety-related behaviour is decreased in pregnant and lactating rodents tested in the open field and on the elevated plus maze (Fleming and Luebke, 1981, Neumann, 2001, Wartella et al., 2003, Hillerer et al., 2012).

The brain OXT and PRL systems have also been shown to be involved in the display of the peripartum-associated maternal calmness. Thus, OXT antagonist intracerebroventricular infusion was reported to increase anxiety-related behaviour in pregnant (PD 20-21) and lactating (lactation day (LD) 7-11) rats tested on the elevated plus maze with treated rats showing lower number of entries and less total time spent on the open arm (Neumann et al., 2000b). Behavioural effects of central injected antisense against the PRL receptor followed the same trend. Indeed, lactating rats with down-regulated PRL receptor displayed increased anxiety-related behaviour when tested on the elevated plus maze (Torner et al., 2002). In line with these animal findings, women who breastfeed were shown to have increased plasma levels of PRL and OXT (Chiodera et al.,

1991, Nissen et al., 1996); higher plasma levels that are hypothesised to mirror an increase in the central release of these neuropeptides (Neumann and Landgraf, 1989, Neumann et al., 1993, Torner et al., 2004). Intriguingly, breastfeeding mothers were shown to respond with lower ACTH and CORT rise compared to non-breastfeeding mothers after graded treadmill exercise (Altemus et al., 1995). Breastfeeding was even shown to affect the hormonal response to a brief psychosocial stressor exposure. In detail, women who breastfed their infants for 15 minutes during the 30 minutes period prior to stress exposure reported attenuated plasma ACTH and CORT levels compared to women who were just holding their babies (Heinrichs et al., 2001). PRL levels in women just prior to delivery were reported to negatively and significantly correlate with the scores on the Hamilton anxiety scale leading to the hypothesis that high PRL levels may lead to lower anxiety level in lactating women (Asher et al., 1995). Also high OXT levels were reported to be fundamental for healthy mother-infant interaction, an important component of the maternal behaviour that was shown to be impaired in mothers suffering from postpartum mood disturbances (Hornstein et al., 2006). Specifically, mothers with secure attachment to their infants had higher peripheral plasma OXT response to physical contact with the child (Strathearn et al., 2009) while low plasma OXT concentration at mid-pregnancy predicted PPD symptoms assessed two weeks after delivery (Skrundz et al., 2011). Several animal studies focused on the OXT system involvement for the mother-offspring interaction and on how maternal stress impacts on the OXT system may mirror deficit in maternal behaviour. Champagne and colleagues reported that rat dams from a line selectively bred to show high frequency of licking and grooming of the pups had higher levels of OXT receptor in distinct brain regions known to play a central role in the regulation of maternal behaviour, like the bed nucleus of the stria terminalis, the medial preoptic area and the central amygdala compared to low licking and grooming mothers (Champagne et al., 2001). Interestingly, exposure of the high licking and grooming mothers to chronic restraint stress exposure during pregnancy resulted in OXT receptor

expression comparable to those of low licking and grooming mothers, change that was paralleled by a decrease in the licking and grooming behaviour (Champagne and Meaney, 2006).

1.5. Peripartum-associated mood and anxiety disorders and maternal obesity

While decreased anxiety and stress reactivity are common peripartum changes, the postpartum period also represents a period of susceptibility to develop mood and anxiety disorders. Indeed, in a large subset of mothers, this phase represents a risk period for the development of such pathologies particularly damaging because they affect not only the well-being of the mother, but also negatively impact the development of the infant as well, increasing the likelihood of them developing a psychiatric disorder in later life (Weinstock, 2001, Newport et al., 2002, Davalos et al., 2012, Perani and Slattery, 2014). Several postpartum mood and anxiety disorders have been described and include PPD, postpartum anxiety and postpartum psychosis.

1.5.1. Postpartum mood disorders

The most common, and least severe, postpartum mood disorder is postpartum blues, which affects between 30-75% of mothers within the first two weeks after delivery. The “blues” are characterized by changeable mood with crying and other symptoms including feeling of confusion and sleep disturbances often observed. Postpartum blues often resolve spontaneously within two weeks without negative consequences for the mother or the child (Seyfried and Marcus, 2003). PPD is more serious for both the mother and offspring (O'Connor et al., 2002, Deave et al., 2008) and represents an episode of major

depression with a specific temporal manifestation, which is still under debate. Currently, the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders sets the first sixth months postpartum as the period at risk for developing PPD, which occurs in 10-22 % of mothers; often as a result of long-lasting blues episode (O'Hara and McCabe, 2013). The majority of the symptomatology associated with PPD is indistinguishable to other depressive episodes (Cooper et al., 1988), however, a distinguishing feature is the loss of interest in the infant, which can often present as the mother finding infant stimuli aversive (Adamakos et al., 1986, Bifulco et al., 2004). Psychological and/or pharmacological treatment is often required for these disorders amid several concerns regarding the use of antidepressants during breastfeeding (O'Hara and McCabe, 2013). Breastfeeding importance during the first six postnatal months discouraged nursing interruption considering that the newer antidepressant have been shown to transfer to the offspring at low amount and they are not associated with serious adverse events so far although long term neurodevelopmental effects are not yet well characterized (Chad et al., 2013). Moreover, pharmacological treatment may improve maternal mood which, in turn, positively impacts on the infants (Gentile, 2005). Maternal anxiety during the postpartum period may also be pathological (Lonstein, 2007), when there is no specific reason for its appearance, or when the intensity and duration of the symptoms are disproportional to the situation that elicits the episode (Correia and Linhares, 2007) and is often observed in woman with PPD (Ross et al., 2003). However, there is evidence to suggest that it can exist as a separate disorder and it has been speculated that it may even display a higher prevalence than PPD (Wenzel et al., 2005). Therefore, it is important to consider and study these two pathologies separately. Mothers with postpartum anxiety show bidirectional parenting styles with one subset showing reduced coping and reactivity to the infant and a second group showing a highly protective maternal style, often termed “helicopter parenting” (Bridges, 2008). Postpartum psychosis is probably the most serious postpartum illness and has a prevalence of between 0.1 – 0.5 % of mothers. It has been

hypothesised to feature, or even be a subset, of bipolar or schizoaffective disorder and it may culminate with suicide or infanticide, thus, mothers must be hospitalized for their own and their child's well-being (Appleby et al., 1998).

Although described separately, there is substantial overlap in the symptomatology of these disorders (with blues not actually representing a psychiatric disorder), and therefore, they can be considered as it continuum with overlapping symptomatology in terms of diagnosis and treatment. Whilst the direct causes for these disorders unfortunately still remain unclear, predisposing and risk factors have been identified, which are discussed next.

1.5.2. Risk factors for the development of postpartum mood and anxiety disorders

The main predictor for depressive, anxiety or psychotic diseases after delivery is an antenatal episode of the illness (Rich-Edwards et al., 2006, Grant et al., 2008, Topiwala et al., 2012). However, other environmental factors have been shown to increase the likelihood for the development of a postpartum psychiatric disorder, including drug abuse (Beck, 2006, Friedman and Resnick, 2009) and low socioeconomic status (Soderquist et al., 2009). Marital problems have also been described as moderate risk factors for the development of PPD (Beck, 2001), while obstetric pregnancy-related complications like caesarean section, or instrumental delivery, are classified as small risk factors (Warner et al., 1996, Johnstone et al., 2001). One of most convincing, and translational, risk factors is chronic psychosocial stress during the peripartum period (Robertson et al., 2004), which is in agreement with the fact that women exhibit a higher susceptibility to stress-related illnesses, such as mood and anxiety disorders than men, in general (Kessler, 2003). In

keeping, emotional support during pregnancy has been shown to negatively correlate with depression (Beck, 1996). Finally, the dramatic fluctuation in sex steroid levels and in HPA axis activity that occur across the peripartum period have been speculated to play a role in these postpartum psychiatric disorders (Bloch et al., 2000, Bloch et al., 2005, Hillerer et al., 2012). Growing evidence suggests that obese mothers are more prone to develop postpartum mood disorders or anxiety. However, our knowledge about the mechanisms underlying this important comorbidity is limited.

1.5.3. Obesity and HPA axis function

Obesity and overweight are among the most important public-health problems because they impact on a variety of physiological processes. Obese patients have reduced life expectancy often because of comorbidity with severe chronic pathologies like hypertension, type 2 diabetes, cardiovascular diseases and depression (Haslam and James, 2005, Tchernof and Despres, 2013).

In the last years has become more evident that obesity associates with multiple alterations of the HPA axis that reflect in changes of basal and stress-induced hormonal release (Pasquali et al., 2002, Bornstein et al., 2006). Despites the multitude of studies available in the literature, a clear understanding of HPA axis dysregulation in obesity is still missing. Indeed, several factors such as age, gender or sampling timing may concur to increase results variability thus masking direct obesity effects on ACTH and CORT levels. Nevertheless, several humans studies suggest that, although basal CORT plasma is not altered in obese patients, the CORT response to stress or food intake is enhanced (Marin et al., 1992, Pasquali et al., 2002). On the other hand, a recent study from Kjölhede and colleagues reported that obese and overweight children have lower CORT levels than

normal weight children during the day (Kjolhede et al., 2013). If in humans basal CORT seems to be unaffected in obese patients or even lower whereas the CORT increase after stress exposure is exaggerated, results from animals studies often diverge. Male rats fed with a high-fat (20% fat) diet were reported to display higher basal CORT levels compared to control rat (4% fat) seven days after fat diet onset and remained elevated up to 21 days while ACTH and CORT were elevated before, during and after restraint stress (Tannenbaum et al., 1997). Another study reported no differences in basal CORT when plasma measures took place sixteen weeks after the beginning of the diet regimen consisting of standard chow (12% fat) for the control group and highly palatable cafeteria-style diet (32% fat) for the high-fat diet experimental group. On the other hand, in the same study two hours restraint stress elicited higher plasma CORT 30 minutes after the stress termination (South et al., 2012). Differences in the experimental conditions may once again underlie the discrepancy from the results of the previously mentioned studies.

HPA axis dysregulation is hypothesised to underlie the comorbidity between obesity and mood and anxiety disorders (Bornstein et al., 2006). Interestingly, epidemiological studies showed for example that adolescents suffering from depression are more prone to develop obesity and obese individuals present higher risk to develop depression than lean subjects (Bornstein et al., 2006, Kyrou et al., 2006). Patients suffering from Cushing's syndrome, an endocrine syndrome characterized by several features, among them obesity and higher CORT resulting from an exaggerated HPA axis activation or exogenous CORT administration, often concomitantly suffer from mood disorders like major depression (Reincke, 2000, Sonino et al., 2010). Intriguingly, such Cushing's syndrome-associated mood disorders are successfully treated with removal of the pituitary and/or adrenal tumors or by CORT synthesis inhibitors (Engelhardt and Weber, 1994).

1.5.4. Maternal obesity

Particularly during the peripartum period, maternal obesity, overweight or even excessive weight gain during pregnancy have been shown to negatively impact on several pregnancy outcome. Specifically, the risk for hypertensive disorders, diabetes, obstetric complications and delayed lactation is increased in obese mothers (Hernandez et al., 2012, Scott-Pillai et al., 2013). Moreover, the negative programming effect of maternal obesity on offspring development and emotionality later in life is well characterized (Rodriguez et al., 2012, Volpato et al., 2012, O'Reilly and Reynolds, 2013). Interestingly, numerous humans studies reported that even during the peripartum period obesity may correlate with mood and anxiety disorders. Overweight women were shown to be at risk for elevated anxiety at 4-month and depressive symptoms at both 4- and 14-month postpartum. Moreover, in the same cohort, 4-month body mass index (BMI) predicted 14-month postpartum depressive symptoms (Carter et al., 2000). Another human study observed that in obese pregnant women the levels of state anxiety significantly increased from the first to the third trimester whereas this parameter remained constant throughout pregnancy in normal-weight women (Bogaerts et al., 2013).

1.5.5. Animal models to study postpartum mood and anxiety disorders

1.5.5.1. Repeated stress based approaches

Mood disorders are often characterized by altered HPA axis activity (Ising et al., 2007). Indeed, a subgroup of depressed patients showed disturbed CORT diurnal rhythms and increased resistance to the feedback action of CORT (Herbert, 2013). More pertinently, mothers suffering from PPD have been reported to display an attenuated physiological

morning rise in CORT (Taylor et al., 2009) and a lack of correlation between ACTH and CORT release elicited by maximal treadmill exercise, such as observed in healthy individuals (Jolley and Betrus, 2007).

Therefore, animal models, designed to interfere with HPA axis function during the postpartum period, have been extensively utilized to determine whether the physiological normal alterations in the maternal HPA axis function, and/or their perturbation, may be associated with postpartum mood and anxiety disorders. These studies are often designed to repeatedly expose pregnant animals to repeated stressful events and conditions, stress that, as mentioned above, is one of the most prominent risk factors for the development of postpartum mood disorders. However, the vast majority of studies in which maternal stress is employed are used almost exclusively to examine the consequences of early-life stress (e.g. gestational and/or pre-weaning) on the offspring given the association between early trauma and increased risk for anxiety and depression disorders, schizophrenia and substance abuse in later life (for reviews see (Weinstock, 2001, Meaney et al., 2002, Heim et al., 2004)). Indeed, maternal stress may impact on the offspring directly, when stress is performed during pregnancy and therefore affect prenatal environment or indirectly, when maternal physiology or behaviour is altered. There is also the complication that different stressors are effective in male and female rodents, which is particularly true of social stress paradigms that are believed to more accurately mimic the human situation (Cryan and Slattery, 2007, Reber, 2012). For example, while resident-intruder paradigms work well in male rodents, in order to be effective in females, lactating dams, with their increased level of aggression have to be employed (Neumann et al., 2001, Neumann et al., 2005). Physical stressors, such as repeated restraint stress or chronic mild stress paradigms have been shown to be effective stressors during the peripartum period (Hillerer et al., 2011).

One of the most examined systems following peripartal stress exposure is the HPA axis for obvious reasons. While these studies consistently reveal changes in the normal peripartum-associated adaptations, these differ depending on the stressor and species used. Mice that were subjected to a chronic mild stress paradigm throughout pregnancy were shown to have elevated CORT (and oestrogen levels) when assessed at mid-pregnancy, however this increase was no longer significant in lactation compared with controls (Misdrahi et al., 2005). Similarly, exposure to a psychosocial stress model designed in our laboratory, which combined alternating days of restraint stress and days of overcrowding with unknown females between pregnancy days 4 and 16, prevented the lactation-associated plasma basal hyper-CORT levels in mid-lactation in rat dams (Hillerer et al., 2011). Interestingly, this alteration in CORT was not accompanied by a difference in ACTH levels, which is in keeping with the findings of mothers with PPD who lack the CORT awaking response and show an imbalance in stress-induced ACTH and CORT levels (Jolley et al., Taylor et al., 2009). While the stress paradigm established in our group did not affect lactation-associated stress hyporesponsiveness, repeated strobe-light stress throughout pregnancy and lactation resulted in an enhanced CORT response to stress in the afternoon compared to the morning (Leonhardt et al., 2007). Further, rats exposed to repeated restraint stress between pregnancy day 10 – 20 were observed to exhibit enhanced acute stress-induced ACTH and CORT secretion in lactation (Smith et al., 2004). Therefore, it appears that depending on the stress paradigm employed, different effects on peripartum-associated HPA axis adaptations can be observed.

Repeated stress has also been shown to affect maternal care, with repeated restraint stress between pregnancy days 4-14 decreasing both maternal aggression and pup retrieval in mice (Maestripieri et al., 1991). The latter effect was also observed following a chronic mild stress paradigm throughout the whole pregnancy period, in which mice were exposed to a variety of mild stressors, such as cage tilt and alterations in the light/dark

cycle (Pardon et al., 2000). Basic maternal care in the home-cage was not affected by this paradigm but chronic stress exposure has been shown to bi-directionally affect kyphotic nursing, the most active form of nursing in rodents. Thus, repeated restraint stress, as well as exposure to CORT, has been shown to decrease this form of nursing (Smith et al., 2004, Brummelte and Galea). Exposure of dams to a daily chronic social stress paradigm, comprised of placing a similar sized male into the dam's home-cage between postnatal days 2-16, was also shown to result in reduced maternal care compared with non-stressed dams (Nephew and Bridges, 2011, Murgatroyd and Nephew, 2013). Further, Kurata et al. (Kurata et al., 2009) subjected dams to an inescapable shock on postnatal day 3 and assessed the number of escape attempts 24h later and classified dams as non-learned helplessness or learned helplessness. Interestingly, learned helplessness dams were shown to have reduced levels of active nursing behaviour until mid-lactation, which suggests that the learned helplessness paradigm may be a useful model to study PPD. Similar findings have also been observed in gorillas and baboons, where high perceived levels of stress are associated with decrease care of the infants (Bahr et al., 1998, Brent et al., 2002). In the psychosocial stress paradigm established in our laboratory, however, stressed dams displayed increased kyphotic nursing in the early lactation phase (Hillerer et al., 2011). Chronic stress was reported to increase both anxiety- and depression-like behaviour in the postpartum period. Exposure to the pregnancy chronic stress model from our group prevented lactation-associated anxiolysis (Hillerer et al., 2012), behavioural readout that was confirmed even when dams were tested 26 days after exposure to repeated restraint stress performed during the last week of pregnancy (Darnaudery et al., 2004). Alternation of restraint and novel environment stress from early-to-late pregnancy increased anxiety-related behaviour when tested on postnatal day 6 (Maestripieri et al., 1991). Repeated restraint stress increased depressive-like behaviour when animals were tested on postpartum day three, four (Smith et al., 2004) or 22 (O'Mahony et al., 2006) but not 35 and 36 post-stress

(Darnaudery et al., 2004) while one study reported unaltered depressive-like behaviour when pregnant animals were tested on day three post-stress (Pawluski et al., 2011). Finally, repeated restraint stress throughout the last week of pregnancy has been shown to prevent the increase in spatial memory observed in mothers 16 months later (Lemaire et al., 2006). This not only suggests that stress during pregnancy has long-lasting consequences for the mother but that more should address which such long-term consequences may arise.

Taken together, chronic stress has repeatedly been shown to affect maternal HPA axis-related adaptations and behaviour. However, the outcome appears to be heavily reliant on the model employed and, therefore, makes comparison across the different studies more complicated.

1.5.5.2. High-fat diet based models

It is now well-established that obesity shares biological underpinnings with HPA axis diseases at several levels (Bornstein et al., 2006); for example obesity has been well-documented to interfere with basal and stress-induced HPA activation (Kyrou et al., 2006). Thus, basal plasma CORT negatively correlates with waist-to-hip circumference ratio in obese women, whereas the stimulation of CORT secretion via administration of an ACTH analogue was augmented, suggesting an increased sensitivity of adrenal responses in obese woman (Marin et al., 1992). Studies have also reported that obese subjects show a paradoxically low levels of plasma CORT, which may be linked with changes in CORT clearance (Marin et al., 1992, Andrew et al., 1998) and metabolism (Stewart et al., 1999). Therefore, recent attempts have focused on the development of animal models to study this multifaceted peripartum comorbidity. A study from Purcell and colleagues (Purcell et

al., 2011) revealed that rat dams fed from the beginning of pregnancy with a high fat diet (60% fat) decreased the time the mothers spent in active nursing. Thus, more studies assessing the effect of high-fat diet / obesity in animal models may help to increase our understanding of postpartum psychiatric disorders; particularly those in relation to obese mothers.

Importantly, although chronic stress and high fat diet exposure have been reported to markedly impact on HPA axis-related hormonal function peripartum, the potential effects on adrenal morphology and function are still unknown despite the adrenals importance for the overall HPA axis function as site of CORT production and particularly when plasma ACTH and CORT disconnect, feature that characterizes several pathological conditions.

1.6. Aims of the thesis

The adrenal glands have longer been hypothesised to undergo plasticity when ACTH and CORT levels disconnect, hormonal feature that characterizes several physiological and pathological conditions (Bornstein et al., 2008) as extensively discussed in Sections1.3.3, 1.4.2 and 1.4.3. Specifically, ACTH and CORT transiently dissociate across the estrous cycle whereas this discrepancy is sustained peripartum (see Section 1.3 and 1.4, respectively). Furthermore, growing evidence suggests that adrenal glands mechanisms might participate to the aetiology of peripartum pathologies that are associated with HPA axis overall dysbalance.

Therefore, with the present thesis I aimed to:

1. Characterize adrenal glands plasticity across the estrous cycle and the peripartum period with a special focus on the pathways involved in cholesterol delivery, important component of the steroidogenic machinery.

2. Investigate whether chronic pregnancy stress and high-fat diet intake interfere with the peripartum-associated adrenal plasticity revealed in the present thesis. After showing that high-fat diet intake prevented most of the changes in adrenal cholesterol delivery occurring at mid-lactation, I further aimed to assess whether these effects associate with altered maternal HPA axis (re)activity and behaviour.

3. Question the translational significance of the present thesis findings. To assess that, I investigated if the changes in adrenal cholesterol delivery observed in the rat are species specific adaptations or whether they occur also in the lactating mouse.

Chapter 2: Adrenal gland plasticity across the estrous cycle and the peripartum period

2.1. Introduction

As stated in the general introduction, HPA axis peripartum plasticity has been extensively documented with the most characterised changes including increased basal levels of plasma CORT and a parallel reduction in the HPA axis response to stress in numerous species (Johnstone et al., 2000, Heinrichs et al., 2001, Kammerer et al., 2002, Deschamps et al., 2003). Interesting, it appears that the basal hyper-CORT occurs independently of changes in the CORT main secretagogue, ACTH (Atkinson and Waddell, 1995). While brain and pituitary adaptations in the CRF, AVP, PRL and OXT systems are known to play a role (for review see (Slattery and Neumann, 2008, Hillerer et al., 2012); see Section 1.4.2.), to my knowledge, any notion about the potential contribution of the adrenal glands to the overall peripartum HPA axis regulation is lacking. Thus, while it has repeatedly speculated that the discrepancy between ACTH and CORT may be mediated by adrenal gland ACTH-R sensitivity, no studies have investigated this hypothesis.

In contrast to the lack of peripartum-associated adrenal studies, changes in adrenal morphology particularly regarding cholesterol delivery pathways have often been associated with altered basal and ACTH-induced CORT release (Uschold-Schmidt et al., 2012, Fuchsl et al., 2013). The events that result in cholesterol availability for steroidogenesis, cholesterol availability that is considered as the rate limiting step for steroidogenesis (Hu et al., 2010), can be classified in three main processes: cholesterol supply to the adrenals, intracellular transfer and metabolism. Changes in cholesterol metabolism can manifest rapidly (within minutes), for example after hormonal stimulation,

or may take longer for example hours (>three hours) or even days when changes in the number of steroidogenic cells are involved. Rapid changes rely mainly on cholesterol availability i.e. from lipid stores, plasma lipids and *de novo* synthesis, while longer changes imply increased gene expression of proteins involved in cholesterol metabolism and in the hormone biosynthetic pathways such as StAR and Cytochrome P450 enzymes (Jefcoate et al., 1992, Sewer and Waterman, 2003, Sewer et al., 2007) (see Section 1.2.2 for full description of cholesterol delivery pathways).

As mentioned above, changes in adrenal cholesterol delivery have already been repeatedly documented to affect CORT release. Thus, lack of the transcription factor BMAL1, an important component of the circadian timing system, was shown to induce adrenal lipid droplets depletion and down-regulation of genes encoding for proteins involved in cholesterol transport (StAR and LDLR). These changes were hypothesised to underlie the hypo-CORT state of these mice (Leliavski et al., 2013).

Furthermore, KO studies have highlighted how ablation of genes coding for proteins like HSL and LDLR impacts on steroidogenesis. Thus, HSL-mediated cholesterol delivery (from SRB1 uptake and lipid droplets recruitment) is believed to be necessary for ACTH-induced CORT production while emerging evidence suggests that LDLR uptake is not involved in acute adrenal steroidogenesis (Kraemer et al., 2002, Kraemer et al., 2007).

The findings mentioned above indicate that adrenal cholesterol delivery pathways represent an highly plastic system that extensively adapts when adrenal homeostasis is threatened. Therefore, taken that the maternal basal hyperCORT state represents a challenge for the adrenal steroidogenic machinery with the cholesterol demand being increased and sustained it is likely that adrenal cholesterol delivery pathways adapt peripartum.

However, any evidence of adrenal plasticity across pregnancy and lactation is lacking.

Peripartum hyperlipidemia, an important adaptations that mirror the changes in the maternal metabolism necessary to face the increased energy demand during the phase of maximal foetal growth (Desoye et al., 1987, Herrera et al., 2006) suggests that adrenal cholesterol supply might be affected. Indeed, in several species, including rodents and humans, plasma cholesterol and triglycerides rise starting at mid-pregnancy.

A potential system underlying maternal basal hyper-CORT is the OXT system taking its highly peripartum activation reflected by high plasma OXT levels under both basal and stress-induced conditions (Neumann et al., 2000b). OXT can influence adrenal steroidogenesis and has been shown to stimulate basal CORT release from dispersed adrenal cells of female rats *in vitro*. This effect has also been reproduced *in vivo*, where a single subcutaneous OXT injection induces an increase in plasma CORT in both, male and female rats (Stachowiak et al., 1995, Petersson et al., 1999). This peripheral effect appears to be in contrast to central OXT effects taking that intracerebroventricular infusion of an OXT antagonist was shown to increase stress-induced plasma ACTH and CORT levels in male and females rats (Neumann et al., 2000a, Neumann et al., 2000b). It is, therefore, plausible that peripartum changes in plasma OXT may contribute to modulate adrenal function.

As site of CORT production, the adrenal glands are hypothesised to actively adapt to mediate CORT rise in the absence of changes in its main secretagogue ACTH plasma levels, neuroendocrine picture that features several physiological and pathological conditions, among them the estrous cycle and the peripartum period (Bornstein et al., 2008). Therefore, the rational of the present study was to determine whether the adrenal gland contribute to the peripartum-associated HPA axis plasticity; particularly in relation to the maternal basal hyper-CORT. To study this, I established a method to assess CORT release from isolated adrenal cells *in vitro* at basal conditions and I characterized the

morphology of all three adrenal cholesterol delivery pathways across the peripartum period, adrenal ACTH-R and OXT-R binding and ACTH-R and OXT mRNA expression.

Pregnant and lactating animals together with mothers four weeks after weaning were tested; the post weaning group was added to verify whether the potential adrenal peripartum-related changes are long-lasting whereas V at D/M were included as control group. To confirm the specificity of the potential adrenal plasticity to the peripartum period and to find the appropriate control group, I first assessed the same parameters across the estrous cycle, since a similar ACTH-CORT dysbalance has been reported as described in Section 1.3.3 (Atkinson and Waddell, 1997).

2.2. Materials and methods

2.2.1. Animals

Female Wistar rats (200 to 250g; Charles River, Sulzfeld, Germany) were left undisturbed for one week after arrival under standard housing (3 to 4 rats per standard rat polycarbonate cage) and environmental (12 hours light - 12 hours dark, lights on at 06:00, 22 ± 1° C, 60 ± 5% humidity) conditions. Water and food (10% fat; ssniff, Germany) were available *ad libitum.* All experimental procedures were approved by the Committee on Animal Health and Care of the local government of the Oberpfalz and complied with international guidelines on ethical use of animals.

2.2.2. Experimental design

To assess adrenal weight and cholesterol delivery pathways plasticity across the estrous cycle and to confirm the fluctuations in plasma ACTH and CORT that have already been shown by others (Atkinson and Waddell, 1997), V animals were killed after four days of single housing and vaginal smears were collected to delineate the three experimental groups: diestrous/metestrous, proestrous and estrous. Animals at diestrous and metestrous were included in the same experimental group taken that HPA axis- and reproduction-associated hormonal profile is similar during these phases (Viau and Meaney, 1991). Adrenals and trunk blood were collected and processed as described below. To avoid masking of side-specific adaptation (Droste et al., 2003), potential differences between left and right adrenals were investigated at first. Thus, after showing that none of the parameter studied differed between left and right adrenals, estrous cycle influence on adrenal lipid droplets was assessed in the left adrenal whereas protein expression analysis was performed in the right adrenal for each animal. A separate

cohort of five V was used to establish a protocol of adrenal cells incubation *in vitro* to assess CORT release at basal conditions.

For the investigation of peripartum-associated changes in plasma ACTH and CORT, in adrenal cholesterol delivery pathways, ACTH-R binding and OXT mRNA expression, Virgins (V) at diestrous/metestrous together with animals at pregnancy day (PD) 4 , PD 13, PD 21, lactation day (LD) 8 and 4 weeks post weaning (PW) were studied (Fig. 12; animals that were one day in advance or one day later the designed time point were also included). V at diestrous/metestrous were included in the study as control group; V at proestrous and estrous were excluded because evidence of HPA axis-associated plasticity during these phases of the estrous cycle were identified through the above mentioned study. Mating was timed to allow the killing of all the animals over two days to minimize environmental influences on the parameter of interest. Each parameter was assessed in left and right adrenal separately; however, left and right outcomes from each animal were pooled because no differences between left and right measures were detected. Adrenal ACTH-R mRNA, OXT-R binding and *in vitro* CORT production from isolated adrenal cells were assessed in a separate cohort of V, PD 21 and LD 8 rats.

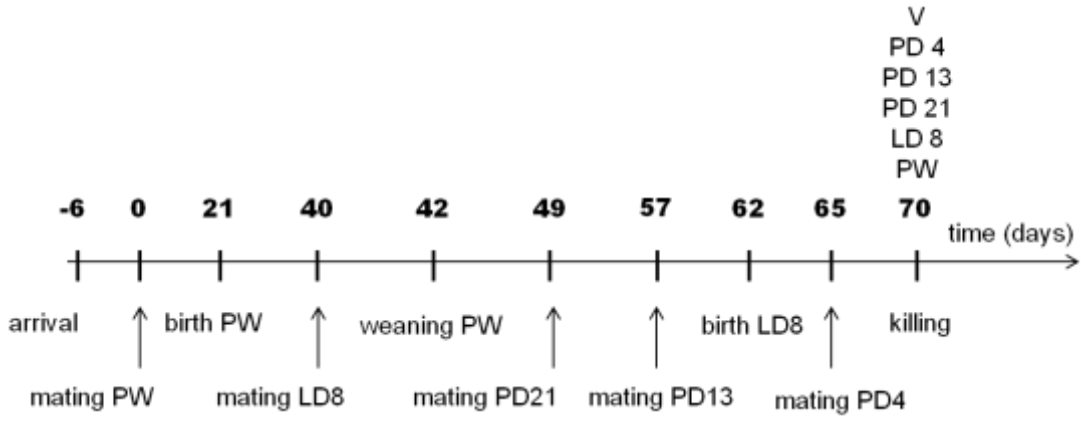


Figure 12. Peripartum plasticity in adrenal cholesterol delivery: experimental design

Animals were mated to allow killing of the animals over two days. Females that did not become pregnant were assigned to the virgin (V) group. Animals at four week post weaning (PW), lactation day (LD) 8, pregnancy day (PD) 21, PD 13, PD 4 and V were included in the study. Each pregnant animal was single housed on PD 17 and, after birth, pups were culled to eight (four males, four females). For the PW group, pups were weaned 21 days after birth and mothers returned group housed until four days before killing when they were single housed.

N group numbers for the investigated parameters across the estrous cycle (table 1 and 2) and the peripartum period (table 3) are reported in the following tables.

Table 1. Side effects on adrenal lipid droplets and protein expression in virgin animals: n numbers

	Left	Right
Adrenal LDLR	12	12
Adrenal SRB1	12	12
Adrenal HMGCR	12	12
Adrenal ACAT	12	12
Adrenal HSL	12	12
Adrenal lipid droplets	6	6

Left and right differences in adrenal protein levels were assessed in four virgins at diestrous/metestrous, four at proestrous and four at estrous. Side differences in adrenal lipid droplets were assessed in two virgins at diestrous/metestrous, two at proestrous and two at estrous

Table 2. Estrous cycle-associated hormonal changes and adrenal plasticity: n numbers

	Diestrous/Metestrous	Proestrous	Estrous
Plasma ACTH	6	6	4
Plasma CORT	5[a]	5[a]	4[b]
Adrenal HSL	4	5	4
Adrenal LDLR	4	5	4
Adrenal SRB1	4	5	4
Adrenal HMGCR	4	5	4
Adrenal ACAT	4	5	4
Adrenal lipid droplets	6	6	6

a) One value excluded due to an Elisa problem

b) Two values excluded due to an Elisa problem

Table 3. Peripartum-associated hormonal changes and adrenal plasticity: n numbers

	V	PD 4	PD 13	PD 21	LD 8	PW
Plasma ACTH	9	5	8	7	8[a]	n.a.
Plasma CORT	8[a]	4[a]	6[b]	7	9	n.a.
Cells CORT release	6	n.a.	n.a.	6	7	n.a.
Adrenal weight	8	5	8	7	9	8
Adrenal ACTH-R mRNA	8	n.a.	n.a.	8	8	n.a.
Adrenal ACTH RAR	4	7	5	5	7	n.a.
Adrenal OXT mRNA	6	5	4	5	5	5
Adrenal OXT RAR	8	n.a.	n.a.	8	8	n.a.
Adrenal lipid droplets	8	4[a]	8	7	9	6
Adrenal LDLR	6	4	5	5	5	4
Adrenal SRB1	3	5	7	7	6	5
Adrenal HMGCR	6	4	6	4	4	4
Adrenal ACAT	2	4	6	7	7	4
Adrenal HSL	2	3	5	7	6	4
Adrenal TH	2	3	4	5	4	4

a) One outsider (in the V group the female was in proestrous)
b) Two outsiders

2.2.3. Mating and verification of pregnancy

Female rats were mated with an experience male (two to three females per male), and pregnancy was verified by vaginal smears (designated PD 0). To rule out any possible effect of mating on subsequent readouts, non-pregnant rats were assumed to be nulliparous (equates to V in the following text). After parturition, defined as LD 0, pups were culled to four females and four males per litter as it has been shown that litter

composition can alter maternal behaviour (Bowers et al., 2013). For the peripartum experiment, animals were single housed 4 days before tissue collection with the exception of LD 8 animals, which were single housed 4 days before predicted parturition (PD 17) to allow undisturbed birth and nursing of the offspring.

2.2.4. Killing - Blood and organ preparation

Animals were decapitated between 08:00 and 11:00 over two days; trunk blood was centrifuged (5000 rcf, 10 min, 4°C), and plasma stored in aliquots at -20°C until ACTH and CORT were measured via enzyme-linked immunosorbent assay (ELISA) using commercially available kits (IBL International GmbH, Germany – CORT kit assay range: 1.63-240 nmol/L, ACTH kit assay sensitivity: 0.22 pg/mL). Each adrenal gland, after pruning from fat was weighed and cut in halves with a scalpel. One half was embedded in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, The Netherlands) and series of five 16 µm cryo-sections from the mid-part of the adrenal glands were cut and then thaw-mounted onto pre-coated slides (slices containing both adrenal cortex and medulla 6 sections per slide; SuperFrost Plus; Germany). The other half was snap-frozen in liquid nitrogen and stored at -80° C until proteins isolation for western blot analysis.

2.2.5. Oil red lipid staining

Adrenal cryo-sections were stained with Oil-red-O (Sigma) for lipids vesicles quantification as previously described (Ramirez-Zacarias et al., 1992, Fuchsl et al., 2013). Six slices per adrenal were stained, photographed and quantified as following described. Each adrenal slide was considered as divided in four areas spreading from the central medulla to the capsule through two imaginary lines crossing in the middle of the adrenal medulla. From each area, a microscopic image at 5x magnification was collected using the Leica V4 image acquisition program. The stained area (mm^2) and the adrenal cortex area

(mm^2) were measured using Leica FW4000 software (Leika Microsystems, Germany) and the ratio between the two was calculated. The area stained was expressed as percentage of the adrenal cortex (relative lipids content per cortex area). The adrenal medulla and glomerulosa, both easily distinguishable by eye (Pignatelli et al., 1998), were excluded from the measurement of the adrenal stained and cortex areas allowing specific quantification of the lipid droplets amount in adrenal areas involved in CORT synthesis, the fasciculata and reticularis.

2.2.6. ACTH and OXT receptor autoradiography (RAR)

ACTH and OXT RAR was performed using a linear ACTH-R antagonist [^{125}I]ACTH, (1-39) Tyr^{23} and $^{125}Id(CH_2)_5[Tyr(Me)^2\text{-}Tyr\text{-}NH_2]_9$-OVT as tracer (PerkinElmer, USA), respectively. Briefly, six adrenal 16 µm cryocut sections per animal were thawed, dried and fixed for two minutes in 0.1% paraformaldehyde at room temperature. After two washing steps (10 minutes each) in 50 mM Tris, slices were exposed for 60 minutes to the tracer buffer (50 mM tracer, 10 mM MgCl2, 0.1% BSA) and then washed 3 times in Tris 50 mM, $MgCl_2$ 10 mM buffer. Slides were then dipped in water, air-dried and exposed for 90 days and 4 hours to Biomax MR films (Kodak, France) for ACTH and OXT, respectively. ImageJ 1.47 program was used to take photographs and to measure the receptor binding that was expressed as grey density and calculated for each adrenal by taking the mean of four to six sections per adrenal. Data were adjusted subtracting the background signal to control for non-specific binding.

2.2.7. ACTH-R and OXT in situ hybridization

Deep frozen adrenal slices were brought to room temperature and fixed in 4% paraformaldehyde for ten minutes. The slides were washed in PBS twice and were fixed for 5 minutes in chloroform. Slides were pre-treated with acetanhydride 0.25% in

triethanolamine 0.1 M to reduce background staining, to inactivate RNases and to produce a stronger signal. Dehydration was performed with ethanol solutions in different concentrations (70-100%) to increase the oligonucleotides concentration.

Before the actual hybridization a pre-hybridization was performed by exposing the slides to a pre-hybridization buffer; the incubation was performed at 50°C and lasted two hours. t-RNA present in the pre-hybridization buffer bound free bases unspecific and the unspecific base-pair bonds were removed *via* two wash steps (two times with sodium chloride / sodium citrate buffer and with 70/ 95% ethanol). For the hybridization the dried slides were covered with the hybridization solution overnight and incubated at 50º C. Besides the ^{35}S -tailed oligonucleotide probe that was applied to each section at a concentration of 10^6 cpm per slide, the hybridization solution contained t-RNA at lower concentration than in the pre-hybridization buffer and formamide and DTT that decrease the thermal stability of the bonds and, therefore, enables to carry out the hybridization at lower temperature. The following day the slides were washed with 50°C warm sodium chloride /sodium citrate buffer four times for 15 minutes on a shaker and cooled down to room temperature at the end. The monovalent cations of the sodium chloride / sodium citrate buffer interact with the phosphate groups of the RNA, decreasing the electrostatic interactions between the oligonucleotide and the specific nucleotide probe increasing the stringency of the hybridization. Under highly stringent conditions the hybridization is more specific and, *vice versa,* low stringency would result in a high number of false positives. At the end, one final washing step with ethanol solutions in different concentrations was accomplished before drying the slices to remove unbound probe or probe which has loosely bound to imperfectly matched sequences. To detect the radioactivity the slides were covered by films for four hours and developed. Imaging and receptor density quantification as gray density was carried out with the ImageJ program as described in the previous section for RAR.

2.2.8. Western blot analysis

Adrenal proteins were isolated *via* lysis buffer (50mM Hepes, 250mM NaCl, 0.5mM EDTA, 0.5% Igepal) supplemented with protease inhibitor cocktail (Roche, Switzerland) and the protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). 25µg from each protein extract were separated via electrophoresis performed using 10% sodium dodecyl sulphate-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, USA). Tris-buffered saline/0.1% Tween-20 (TBST, pH 7.6) supplemented with 5% milk powder was used to block the membranes (one hour at room temperature) and to dilute primary antibodies. Blots were incubated over night at 4°C with the specific primary antibody at 1:1000 dilution against LDLR, SRB1 (Abcam), HMGCR, ACAT (Santa Cruz), HSL (Cell Signalling) and at 1:10000 against TH (Millipore). For secondary antibody incubation, membranes were incubated at room temperature for one hour with peroxidase-conjugated anti-rabbit IgG (Cell signalling) diluted 1:1000 in TBST. Blots were washed with TBST after each antibody incubation session. β-tubulin was used as total protein loading control. Therefore, each blot was probed with anti-β-tubulin antibody (Cell Signalling; 1:1000; overnight at 4°C). Secondary antibody incubation was performed as described above. Bands were visualised using ECL western blot detection reagents (GE Healthcare, UK), and images were acquired with the ChemiDoc XRS+ system (Bio-Rad, USA). Each western blot was run in duplicate at the same time; the average of two protein expression values per animal was used for statistical analysis.

2.2.9. Establishment of an adrenal cell *in vitro* incubation protocol

Left adrenal glands from five V rats were homogenized in 5 ml Dulbecco's Modified Eagle medium (DMEM/F-12, Life Technologies, Inc., USA) supplemented with 0.1 % bovine

serum albumin (BSA, Biomol, Germany) after capsule removal taken that previous pilot experiments reported that CORT release did not differ between cells from left and right adrenals (data not shown). Cell homogenates were filtered with 70 µm cell strainers; after centrifugation (800 rpm, 4°C, 10 minutes), supernatants were discarded and pellets re-suspended in 700 µl DMEM/F-12, 0.1% BSA (adapted from (Oitzl et al., 1995, Revsin et al., 2008)). Four pools of 80000 cells *per* animal were incubated at 37°C (95 % O_2, 5 % CO_2). Four incubation durations were tested; one cell pool from each animal was assessed at each time point to assess optimal incubation duration taken that it has been reported that isolated adrenal cells *in vitro* constantly release CORT when functional (Benyamina et al., 1986). After 30, 120, 240 and 360 minutes supernatant from one cell pool from each animal was removed and stored at -20º C until Elisa CORT measurement.

2.2.10. Adrenal cell incubation across the peripartum period

Left adrenals cells from seven V, six PD 21 and seven LD 8 animals were isolated and processed as previously described (see Chapter 2.2.9.). CORT production from isolated adrenal cells has been shown to remain unchanged after four hours incubation and therefore for this experiment one cell pool *per* animal was incubated for 240 minutes.

2.2.11. Statistics

Results were analysed using either a one-way analysis of the variance (ANOVA). Significance, accepted for $P<0.05$, was further analyzed using a Bonferroni *post-hoc* test. Non-parametric Mann Whitney U tests were performed when differences were not detected via ANOVA analysis. Statistical analyses were performed using IBM SPSS (version 21; Chicago, USA).

2.3. Results

2.3.1. ACTH and CORT levels across the estrous cycle and adrenal plasticity

2.3.1.1. Plasma measures

Statistical analysis confirmed that plasma ACTH did not differ across the estrous cycle (Fig. 13 A). On the other hand, plasma CORT varied ($F_{2,13}$=19.27; P<0.001; Fig. 13 B) with CORT being higher at proestrous compared to diestrous/metestrous (P<0.001) and estrous (P<0.01), in agreement with previous findings (Atkinson and Waddell, 1997).

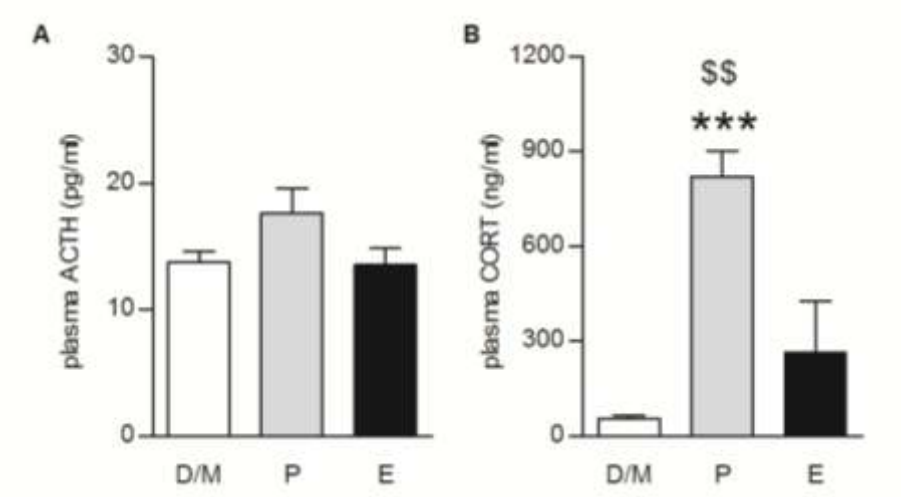


Figure 13. ACTH plasma levels did not differ across the estrous cycle whereas plasma CORT was increased at proestrous (P) compared to virgins at diestrous/metestrous (D/M) or at estrous (E)

Plasma ACTH (A) and CORT (B) levels across the estrous cycle. Data represent mean + standard error of the mean (SEM; n=4-6 per group; see table 2 for the exact n numbers). *** * *** P<0.001 vs. D/M; $ $ P<0.01 vs. E (Bonferroni *post-hoc*).

2.3.1.2. Adrenal weight, protein expression and cortical lipid droplets

Adrenal weight was not affected neither by the side nor by the estrous cycle (diestrous/metestrous n=4: left, 0.039±0.004 right 0.035±0.003; proestrous n=5: left 0.034±0.0019 right 0.034±0.0012; estrous n=4: left 0.036±0.005 right 0.034±0.004). Protein expression and lipid droplets did not differ between left and right adrenal glands (Tab. 4).

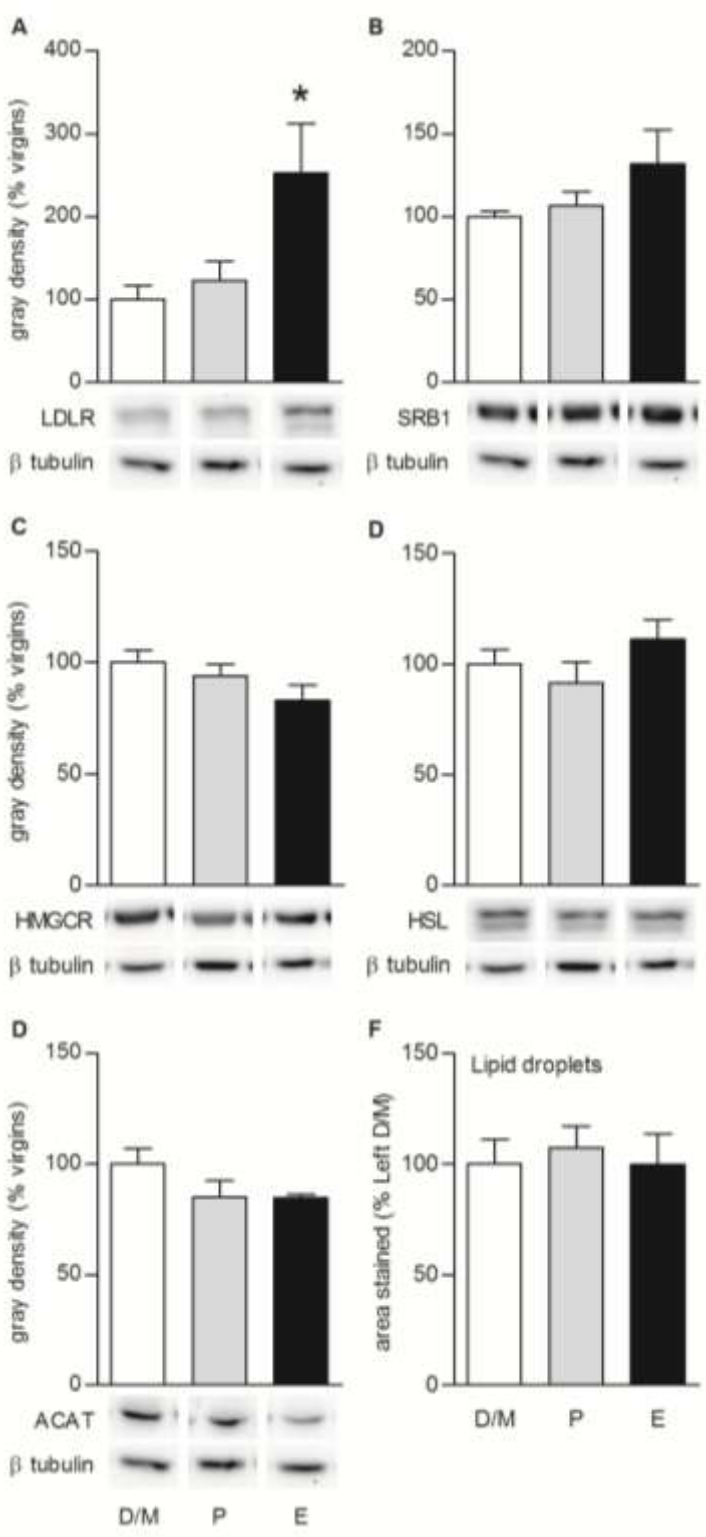
Table 4. Left and right adrenal protein expression and lipid droplets in virgin animals

	Left	Right
Adrenal LDLR	100±40.2	115.6±48.2
Adrenal SRB1	100±19.4	103.7±22.2
Adrenal HMGCR	100±14.1	95.8±11.8
Adrenal ACAT	100±19.5	105.3±24.8
Adrenal HSL	100±29.3	103.9±22.9
Adrenal lipid droplets	100±25.7	73.4±15.7

Results are expressed relative to left measurements

Estrous cycle affected LDLR expression ($F_{2,18}$=4.97; P=0.021; Fig. 14 A) with LDLR being 2.5 fold higher at estrous compared to diestrous/metestrous (P<0.05). A tendency towards higher LDLR at estrous compared to proestrous was observed (P=0.081) while diestrous/metestrous- and proestrous-associated LDLR expression did not differ. No differences in SRB1 (Fig. 14 B), HMGCR (Fig. 14 C); HSL (Fig. 14 D) and ACAT (Fig. 14 E) were observed. Adrenal cholesterol esters stores did not change across the estrous cycle (Fig. 14 F).

Figure 14. Adrenal LDLR protein expression is increased in virgins at estrous (E). Adrenal SRB1, HMGCR, HSL, ACAT protein expression and lipid droplets were not affected by the estrous cycle



Western blot analysis of adrenal LDLR (A), SRB1 (B), HMGCR (C), HSL (D) and ACAT (E) normalized to the loading control β-tubulin and cortical lipid droplets density (F) in virgins at diestrous/metestrous (D/M), proestrous (P) and estrous (E) are reported. Representative blot are reported below the corresponding bars of the protein of interest and the loading control. LDLR expression levels were affected by the estrous cycle. Specifically, the receptor was higher at E compared to D/M (A). None of the other parameter showed plasticity across the estrous cycle. Data represent mean + SEM (n=6-7 per group; see table 2 for the exact n numbers). * $p<0.05$ vs. D/M (Bonferroni *post-hoc*).

2.3.2. Peripartum-associated changes in plasma ACTH and CORT and in vitro CORT production from isolated adrenal cells

2.3.2.1. Plasma CORT and ACTH

A main effect of status on plasma ACTH levels was observed ($F_{4,31}$=3.62; P=0.016; Fig 15 A) and *post-hoc* analysis reported that plasma ACTH was increased in LD 8 dams compared to PD 21 rats (P≤0.01). Status affected also plasma CORT ($F_{4,29}$=5.77; P=0.002; Fig 15 B). LD 8 mothers had higher basal CORT levels compared to V (P<0.01) and to PD 4 rats (P<0.05). Furthermore, separate analysis showed that basal CORT was higher at PD 13 and 21 compared to V (Mann Whitney U; P<0.01 and P<0.05, respectively).

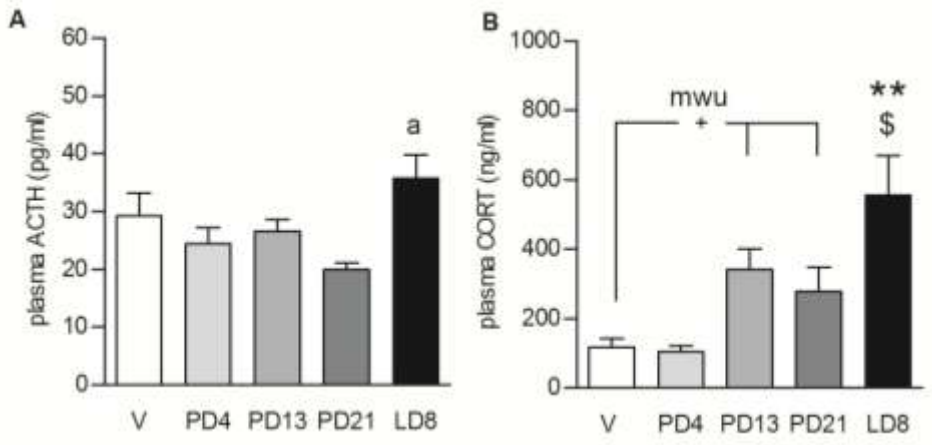


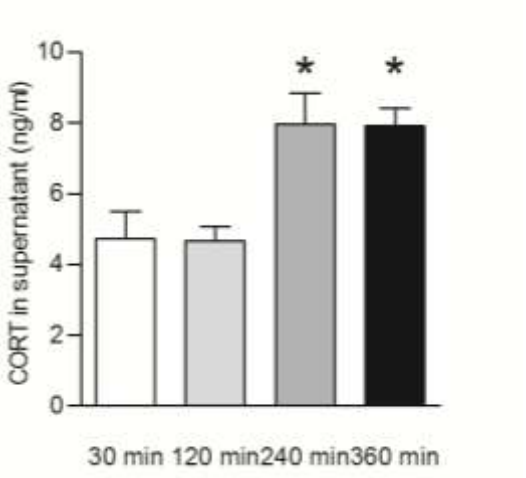
Figure 15. Plasma ACTH was increased on lactation day (LD) 8 whereas CORT levels were high starting on pregnancy day (PD) 13 until LD8

ACTH and CORT levels were measured across the peripartum period in animal at PD 3, pD 13, PD 21, on LD 8 and in virgin rats (V). ACTH levels were higher on LD 8 compared to PD 21 animals but no other difference were observed (A). Basal hyper-CORT was confirmed with CORT levels being high in PD 13, PD 21 and LD 8 animals compared to V (B). Data represent mean + SEM (n=4-9 per group; see table 3 for the exact n numbers). a P≤0.01 vs. PD 21; * * P<0.01 vs. V and $ P<0.05 vs. PD 4 (Bonferroni *post-hoc*); + P<0.05 after Mann Whitney U test (mwu) analysis.

2.3.2.2. CORT release from isolated adrenal cells from V rats *in vitro*

One way ANOVA analysis reported that CORT release in the supernatant from the different pools of 80000 adrenal cells isolated from five V animals was affected by the duration of the incubation ($F_{3,16}$=7.94; P=0.002; Fig. 16). The release after 30 and 120 minutes did not differ. Maximal release was reached after 240 minutes (P<0.05 vs. 30 and 120 minutes) while the release at 360 minutes was comparable to the 240 minutes time point (P<0.05 vs. 30 and 120 minutes).

Figure 16. CORT release from isolated adrenal cells from virgin (V) rats *in vitro* increased after 240 minutes incubation period

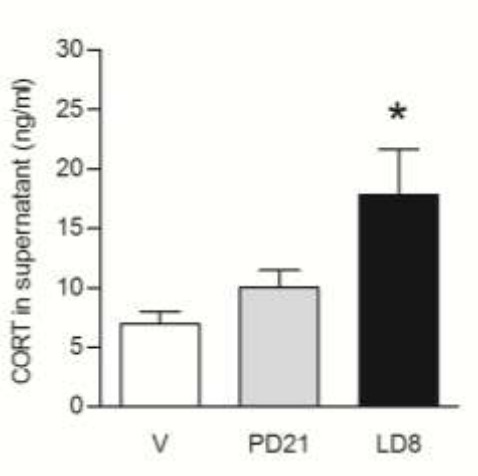


An effect of the duration of the incubation on isolated V adrenal cells was observed on CORT release. Statistical analysis revealed that CORT in supernatant was higher after 240 minutes compared to the release at 30 and 120 minutes. The CORT release did not differ between the 240 and 360 minutes time points. Different cells pooled were incubated in duplicates. Data represent mean + SEM (n=5). * P<0.05 vs. 30 and 120 minutes (Bonferroni *post-hoc*).

2.3.2.3. CORT release from isolated adrenal cells *in vitro* from V, PD 21 and LD 8 animals

One-way ANOVA analysis showed that the reproductive state affected CORT release from isolated adrenal cells *in vitro* ($F_{2,18}$=4.64; P=0.026; Fig. 17). Specifically, the CORT released from LD 8 animals adrenal cells was significantly increased compared to the V related release (P<0.05).

Figure 17. CORT release from isolated adrenal cells from lactation day (LD) 8 dams was increased compared to the releas from virgins (V) and animals at pregnancy day (PD) 21 adrenal cells



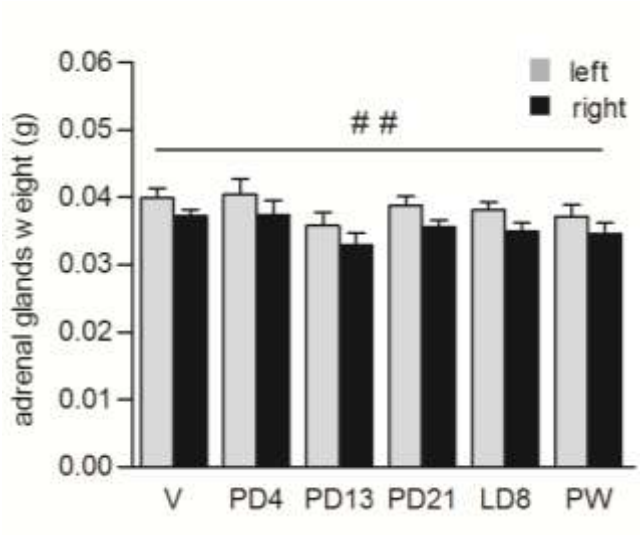
Cells from isolated adrenal glands from LD 8 animals released higher CORT compared to cells from V animals. Data represent mean + SEM (n=6-7; see table 3 for the exact n number). * P<0.05 vs. V (Bonferroni *post-hoc*).

2.3.3. Peripartum-associated adrenal plasticity

2.3.3.1. Adrenal weight

A side effect on adrenal glands weight was observed ($F_{1,78}$=10.11; P=0.002; Fig. 18) whereas the reproductive state did not affect this parameter ($F_{5,78}$=2.27; P=0.056).

Figure 18. Adrenal glands weight did not change across the peripartum period



Adrenal glands weight from virgin (V), animal at pregnancy day (PD) 4, 13, 21, on lactation day (LD) 8 and four weeks post weaning (PW) is represented. A main effect of side on adrenal weight was found while the reproductive state did not affect this parameter. These differences are independent from the reproductive state. Data represent mean + SEM (n=5-9 per group; see table 3 for exact n numbers). # # P<0.01 main ANOVA effect of side.

2.3.3.2. ACTH-R mRNA and autoradiography across pregnancy and lactation

Two-way ANOVA analysis showed that adrenal ACTH-R mRNA expression assessed in V, PD21 and LD 8 animals did not vary as a consequence of status ($F_{2,42}$=2.57; P=0.089) or side ($F_{1,42}$=1.29; P=0.261;Fig. 19 A). Adrenal ACTH-R autoradiography was assessed in all the peripartum groups; however one-way ANOVA analysis reported that the factor status did not affect this parameter ($F_{4,23}$=0.377; P=0.823; Fig. 19 B).

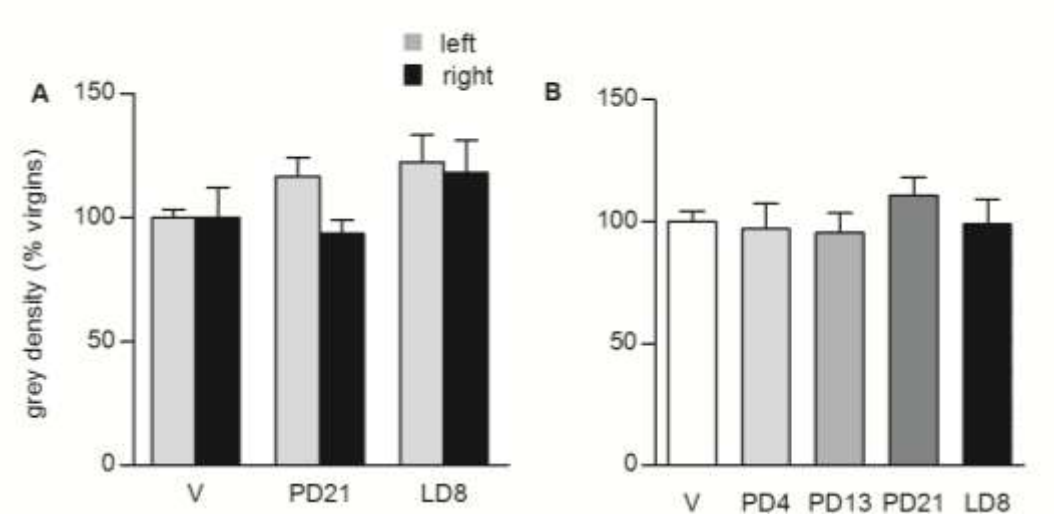


Figure 19. Adrenal ACTH-R mRNA and RAR do not vary across pregnancy and lactation

Adrenal ACTH-R mRNA was quantified *via* in situ hybridisation in virgins (V), and animals at pregnancy day (PD) 21 and lactation day (LD) 8 rats (A). ACTH-RAR was assessed in V and rats at PD 4, PD 13, PD 21 and LD 8 animals (B). Data represent mean + SEM (n=4-8 per group; see table 3 for the exact n number).

2.3.3.3. OXT mRNA and RAR

Two-way ANOVA analysis showed that OXT mRNA content differed between left and right adrenal glands (factor side: $F_{1,52}$=14.2; P<0.001; Fig. 20 A) whereas the status did not influence this parameter ($F_{5,52}$=0.88; P=0.5). Side effects were specific to the V and PD 13 group with right OXT mRNA expression being lower compared to the left one (P<0.01). Furthermore, the factor status affected OXT-R binding ($F_{2,42}$=17.15; P<0.001; Fig. 20 B). Specifically, OXT-R binding was reduced at LD 8 compared to V and to PD 21 in both left (P<0.01) and right adrenals (P<0.05). This parameter was not affected by the adrenal side ($F_{1,42}$=0.19; P=0.658).

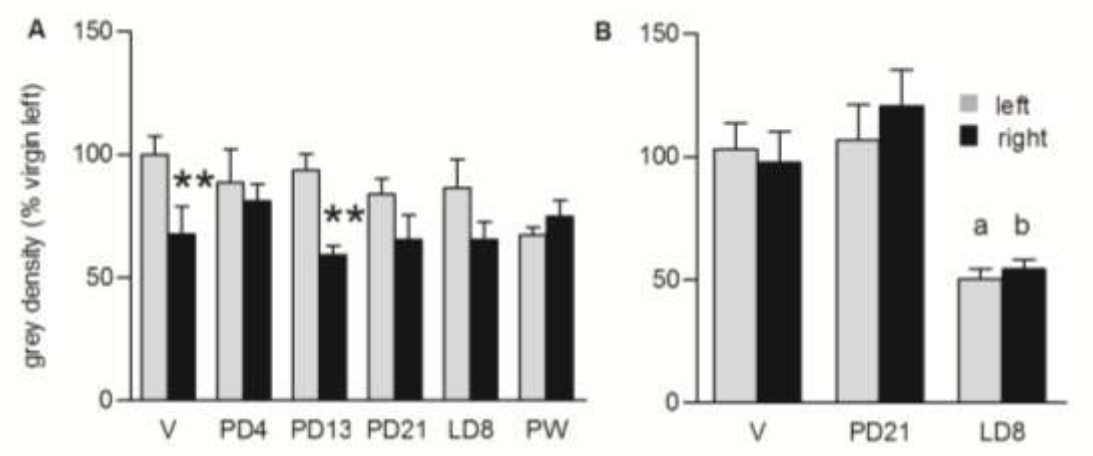


Figure 20. OXT mRNA content is lower in right compared to left adrenals in virgins (V) and animals at mid-pregnancy whereas OXT-R binding is reduced at mid lactation

OXT mRNA content was assessed in V and animals at pregnancy day (PD) 3, PD 13, PD 21, lactation day (LD) 8 and 4 weeks post weaning (PW). OXT mRNA is lower in right compared to left adrenals in virgins V and PD 13 animals (A) whereas measurement of the OXT-R binding in V, PD 21 and LD 8 revealed that this parameter is reduced at LD 8 compared to V and PD 21 in both left and right adrenal glands (B). Data represent mean + SEM (n=5-10 per group; see table 3 for the exact n numbers). * * P <0.01 between left and right adrenals; a P <0.01 vs. V and PD 21 left adrenals; b P <0.05 vs. V and PD 21 right adrenals (Bonferroni *post-hoc*).

2.3.3.4. Adrenal lipid droplets

One-way ANOVA statistical analysis revealed that the reproductive status markedly influenced adrenal cortical lipid droplets ($F_{5,36}$=17.6; P<0.001; Fig. 21 A). Adrenal cholesterol stores were markedly depleted on PD 13 and LD 8 compared to V, PD 4 and PW animals (P<0.001). At LD 8 the stored were reduced also compared to PD 21 animals (P<0.05). At PD 21, lipid droplets were reduced compared to PD 4 (P<0.05) and V (Mann Whitney U; P<0.01).

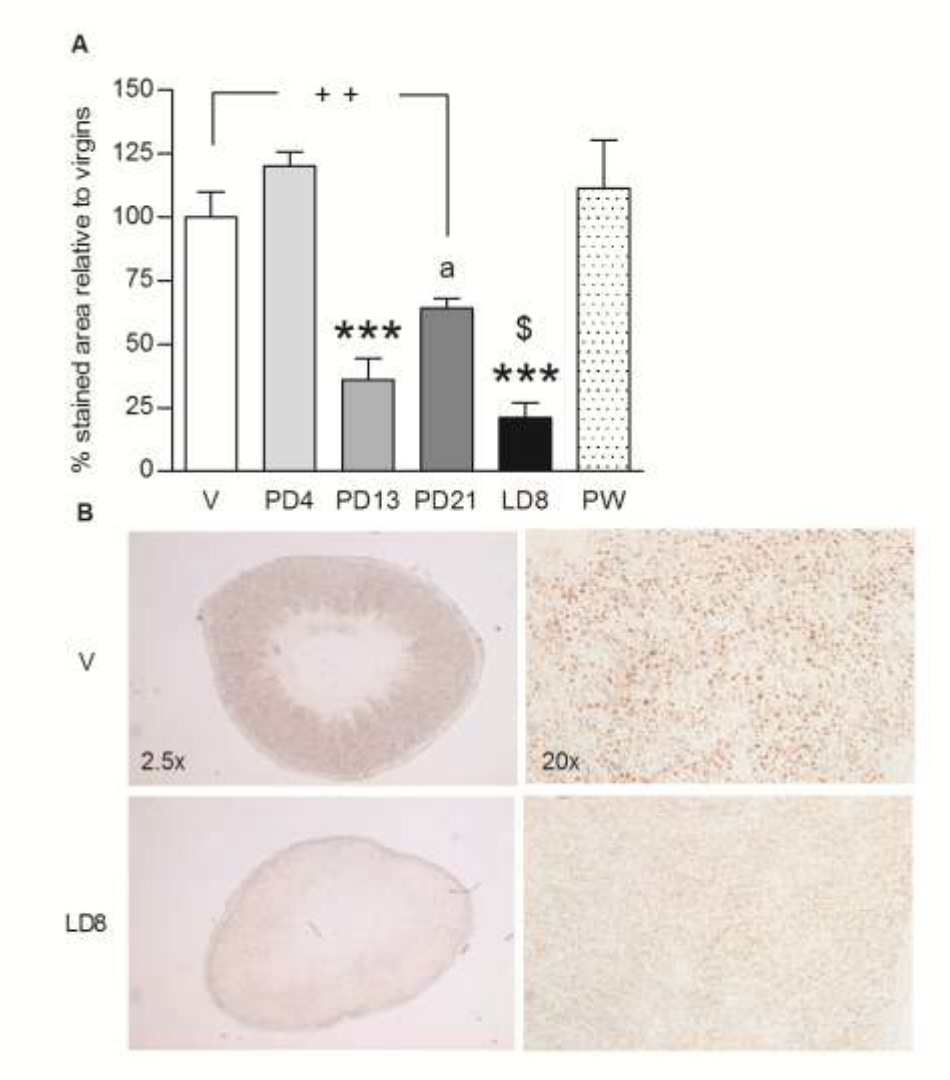


Figure 21. Cortical lipid droplets are markedly depleted in pregnant and lactating animals

Adrenal cortical lipid droplets were quantified in virgins (V) and animals at pregnancy day (PD) 3, PD 13, PD 21, lactation day (LD) 8 and 4 weeks post weaning (PW). Adrenal cholesterol stores are decreased at PD 13, PD 21 and LD 8 compared to V, PD 4 and PW animals (A). Representative pictures from V and LD 8 animals of stained adrenal sections are reported (2.5 and 20x magnification; B). Data represent mean + SEM (n=4-9 per group; see table 3 for the exact n numbers). * * * P <0.001 compared with V, PD 4 and PW animals; $ P<0.05 vs. PD 21; a P<0.05 compared to PD 4 rats (Bonferroni *post-hoc*); + + P<0.01 after Mann Whitney U test (mwu) analysis.

2.3.3.5. Adrenal protein expression

The two main receptors involved in lipid uptake from the plasma, LDLR and SRB1 were affected by the reproductive status (LDLR: $F_{5,23}$=14.4; P<0.001; Fig. 22 A; SRB1: $F_{5,26}$=9.8; P<0.001; Fig. 22 B). LDLR and SRB1 expression levels were increased in LD 8 dams compared to all the others groups (P<0.001 and P<0.01, respectively). Adrenal HMGCR was also affected ($F_{5,22}$=3.12; P=0.028; Fig 22 C); the enzyme was lower at LD 8 compared to PW (P<0.05) and to V (Mann Whitney U; P<0.05). The reproductive status did not affect ACAT ($F_{5,24}$=0.41; P=0.836; Fig 22 D), HSL ($F_{5,21}$=1.66; P=0.187; Fig. 22 E) and TH ($F_{5,16}$=0.49; P=0.780; Fig. 22F).

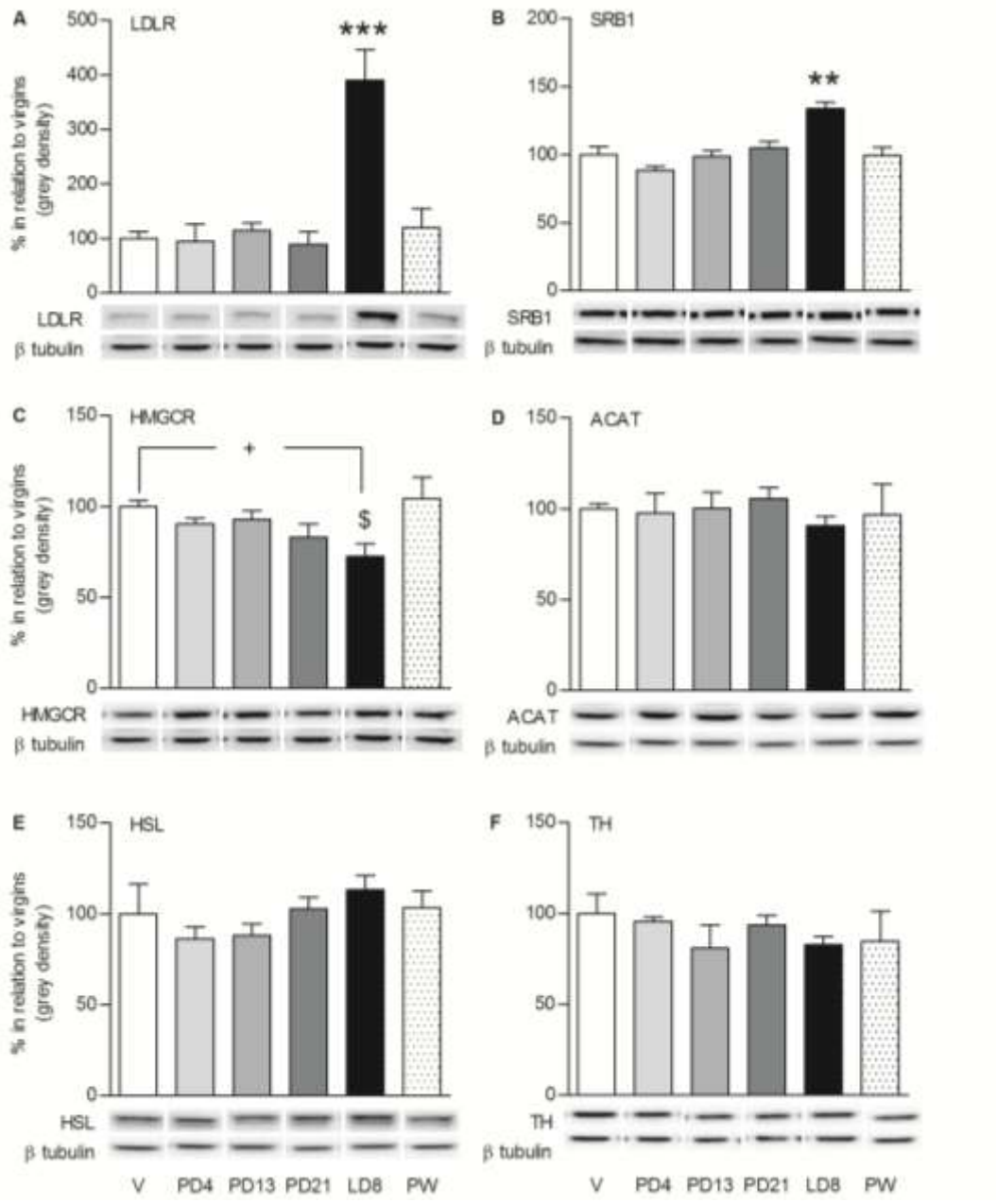


Figure 22. LDLR and SRB1 adrenal expression is increased whereas HMGCR protein levels are lower at mid-lactation

Western blot analysis of adrenal LDLR (A), SRB1 (B), HMGCR(C), ACAT (D), HSL (E), and TH (F) normalized to the loading control β-tubulin in virgins (V) and animals at pregnancy day (PD) 3, PD 13, PD 21, lactation day (LD) 8 and 4 weeks post weaning (PW). LDLR and SRB1 are higher expressed at LD 8 compared to all the other groups while HMGCR levels are lower compared to V and PW animals. Representative blots are shown below the corresponding graphs (upper bands) together with the loading control β-tubulin (lower bands). Data represent mean + SEM (n=3-6 per group; see table 3 for the exact n numbers). * * * P<0.001, * * P<0.01 vs. all the groups; $ <0.05 vs. PW (Bonferroni *post-hoc*); + P <0.05 vs. V after Mann Whitney U test (mwu) analysis.

2.4. Discussion

The aim of the present study was to assess whether the dissociation of plasma ACTH and CORT levels documented across the estrous cycle and the peripartum period are accompanied by evidence of adrenal glands plasticity. Substantial changes in adrenal glands morphology were identified with the present study, particularly regarding adrenal cholesterol delivery pathways. Specifically, adrenal lipid droplets, cholesterol source that is known to be the preferential source recruited in case of acute ACTH-stimulated steroidogenesis, were markedly depleted from PD 13 until LD 8 concomitant with plasma basal hyperCORT. Moreover, adrenal SRB1 and LDLR protein expression was increased whereas HMGCR protein levels were decreased on LD 8. In the present study, isolated adrenal cells from LD 8 animals were reported to reproduce the basal hyperCORT even when isolated *in vitro* suggesting that adrenal plasticity might directly participate to modulate HPA axis activity. Therefore, taken together these findings represent the first evidence of peripartum adrenal plasticity and shed light on the mechanisms that concur to the increased maternal basal adrenocortical activity and stress hyporesponsiveness. Moreover, neither ACTH-R mRNA nor ACTH-R binding were affected across the peripartum period suggesting that other factors beyond ACTH might trigger adrenal changes and basal plasma hyperCORT, at least during pregnancy. The changes observed were specific to the peripartum period taking that four weeks PW all the changes were back to V levels and the estrous cycle-associated adrenal changes were limited to an increase in LDLR expression, increase that was anyway less pronounced when compared to the changes observed on LD8.

My investigation primarily focused on adrenal cholesterol delivery pathways taking that cholesterol availability represents the rate limiting step for steroidogenesis (Hu et al., 2010). Intriguingly, cholesterol delivery pathways adaptations have already been shown

to link to changes in HPA axis basal and stress-induced function in chronically stressed mice that display an exaggerated *in vivo* CORT production following exposure to an heterotypic stress (Uschold-Schmidt et al., 2012, Fuchsl et al., 2013) or in BMAL-1 KO mice that show dysbalance HPA axis activity (Leliavski et al., 2013). Since an increase in basal CORT levels without concomitant rise in ACTH characterises also the proestrous phase of the estrous cycle, a preliminary study was performed to assess adrenal plasticity across the estrous cycle (Atkinson and Waddell, 1997).

Plasma ACTH and CORT across the estrous cycle and the peripartum period

The transient peak in basal plasma CORT levels occurring at proestrous without concomitant changes in plasma ACTH that has already been shown by others (Atkinson and Waddell, 1997) was confirmed with the present study. Also the HPA axis–associated peripartum hormonal changes were confirmed in pregnant and lactating rats when compared with V animals. Specifically, a three- to four-fold increase in plasma CORT was observed in the PD 13, PD 21 and LD 8 groups with plasma ACTH being increased just on LD 8 (Walker et al., 1992, Atkinson and Waddell, 1995, Shanks et al., 1999, Brunton et al., 2008). Taken that peripartum-associated variation in adrenal sensitivity to ACTH has been longer hypothesised to underlie the dissociation of plasma ACTH and CORT, adrenal ACTH-R binding and mRNA was assessed in this study. Intriguingly, neither ACTH-R mRNA nor the ACTH-R binding were affected across the peripartum period. Thus, these findings suggest that ACTH might play just a partial role to sustain peripartum-associated high steroidogenesis; although ACTH involvement cannot be entirely excluded taken that it is still not known whether the pathways downstream to the ACTH-R binding adapt. Therefore, further investigation of potential changes in adrenal sensitivity to ACTH is still needed. *In vitro* methodological approaches like adrenal cells

or explants stimulation with ACTH might address this issue. Unfortunately, despite several attempts, I was not able to establish a functional *in vitro* method to directly verify whether changes in adrenal sensitivity to ACTH underlie peripartum basal hyperCORT.

Dissociation of ACTH and CORT has already been reported to characterize several physiological and pathological conditions and hypothetic central and peripheral factors that might mediate this disconnection have been already identified (Bornstein et al., 2008). An example of "ACTH independent" CORT peak was provided by Choi and colleagues (Choi et al., 2007). They showed that lesion of the anteroventral region of the bed nucleus of the stria terminalis attenuates CORT increase after stress exposure without affecting ACTH levels in male rats (Choi et al., 2007). This finding may be explained hypothesising the existence of brain circuitry that mediate adrenal glands response to ACTH. The sympathetic nervous system is a potential candidate considering that plasma CORT diurnal rhythm was reported to be modulated by adrenal splanchnic innervation that increases adrenal responsivity to ACTH and augments steroidogenesis before the active-phase associate CORT peak in male rats (Ulrich-Lai et al., 2006). In the present study TH expression was measured across the peripartum period given that changes in TH mRNA and protein expression and phosphorylation are believed to mirror potential variation of the sympathetic firing to the adrenal glands (Brooks et al., 1997, Ong et al., 2011). I did not observe any change in TH protein expression; although it is important to remember that TH activity is known to be increased when phosphorylated (Haycock and Wakade, 1992). Therefore, participation of the sympathetic firing cannot be excluded and future studies should address this issue. Nevertheless, to my knowledge, the TH expression measurement reported in the present study represents the first attempt to shed light on the contribution of the sympathetic innervation to the basal adrenocortical activity across pregnancy and lactation. On the other hand, central noradrenergic tone to the PVN has been shown to be reduced during pregnancy and lactation in the rat

(Douglas, 2005), reduction that is hypothesised to concur to the maternal stress hyporesponsiveness at that time. However, adaptations in the neuronal firing activity seem to be highly region-specific taking that measurement of noradrenergic neurons spontaneous firing activity to the locus coeruleus has been reported not to vary between cycling, ovariectomised and PD 17 females (Klink et al., 2002). Therefore, direct investigation of the sympathetic firing plasticity is still needed to clarify its involvement to sustain adrenocortical activity when ACTH and CORT dissociate.

Taken that ACTH and CORT dissociation often manifests depending on the reproductive state, it is highly probable that sex hormones fluctuation may affect adrenal function. Indeed, as mentioned in the introduction (see Section 1.3.3), ovarian steroids involvement in the modulation of adrenocortical activity has already been documented through both, *in vivo* and *in vitro* studies (Nowak et al., 1995, Figueiredo et al., 2007). Figueuredo and colleagues showed that estrogens injections increased stress-induced CORT secretion in female rats despites reduced PVN activation and plasma ACTH, finding that suggests that estrogens might potentiate adrenocortical response to stress. This hypothesis may explain the proestrous-associated CORT increase observed in this study because estrogens are known to peak at proestrous and start to decline in the evening of proestrous (Butcher et al., 1974). Sex hormones might influence adrenal steroidogenesis even during pregnancy when plasma ACTH levels are low and estrogens rise (Bridges, 1984, Brunton and Russell, 2010). On the other hand, the finding reported by others (Walker et al., 1992, Shanks et al., 1999) and confirmed with the present study of increased basal ACTH levels on LD 8, when estrogens are low (Taya and Greenwald, 1982), support this hypothesis and indicate that ACTH may be in place to sustain basal hyperCORT at that time. This hypothesis is strengthened by the *in vitro* finding reported in this chapter about lactation-associated changes in the basal CORT secretion from isolated adrenal cells. Specifically, whilst the basal *in vitro* CORT released by isolated

adrenal cells differed between cells from LD 8 animals and V with “LD 8 cells” showing basal high CORT production that mimics the *in vivo* basal hyperCORT, CORT release from PD 21 animals adrenal cells was not affected. Thus, the involvement of high estrogens levels to sustain basal hyperCORT during pregnancy, estrogens that are missing in the *in vitro* system, might explain these differences. Moreover, these *in vitro* findings highlight that adrenal glands might be fully independent at mid-lactation to sustain basal hyperCORT production without any further external stimuli.

Adrenal changes

The study of adrenal cholesterol delivery pathways plasticity across the estrous cycle and the peripartum period described in the present chapter show that whilst estrous cycle impact on adrenal plasticity is confined to a 2.5-fold increase in LDLR expression at estrous, all adrenal cholesterol delivery pathways adapt across the peripatum period, particularly at LD 8. The estrous cycle associated changes in estrogens may explain the LDLR increase without changes in SRB1 at estrous, phase following the twelve to fourteen hours long prestrous phase when estrogens peak. Indeed, estrogens modulation of adrenal LDLR and SRB1 expression has already been documented by Landschulz and colleagues showing that adrenal LDLR and SRB1 expression are, respectively, four- and ten-fold increased after five days of 17α-ethinyl estradiol subcutaneous injections in the rat (Landschulz et al., 1996). Taken the higher impact of the estradiol injections on the LDLR, it is not surprising that we did not observe changes in the SRB1 after the physiological transient proestrous rise in estrogens. Moreover, evidence of adrenal LDLR and SRB1 expression being differently modulated has already been reported in chronically stressed male mice that show an increase in adrenal SRB1 expression without changes in LDLR (Fuchsl et al., 2013). Intriguingly, whilst pregnancy chronic stress has

already been shown to prevent maternal basal hyperCORT on LD 8 (Hillerer et al., 2011), it is still not known whether prevention of lactation-associated adrenal plasticity might underlie the lack of basal hyperCORT in stressed mothers.

High LDLR and SRB1 adrenal expression at LD 8 suggests that at LD 8, when CORT production is sustained and the adrenal lipid droplets depleted, uptake of extra-adrenal cholesterol is necessary. This hypothesis is supported by the findings that both SRB1 and LDLR levels are high at basal conditions in HSL KO mice, mice that cannot rely on adrenal cholesterol stores recruitment for steroidogenesis (Kraemer et al., 2004).

Adrenal lipid droplets were three- to five-fold decreased already at PD 13, depletion that was not accompanied by any change in plasma lipoproteins receptors or HMGCR expression across pregnancy. These findings suggest that adrenal cholesterol stores might be recruited to sustain the initial phase of basal high CORT production. HSL protein expression was unchanged; however, taken that HSL hydrolytic activity might be modulated *via* phosphorylation mechanisms, increased hydrolytic activity of the enzyme cannot be excluded. Indeed, HSL phosphorylation by PKA is known to increase the hydrolytic activity of the enzyme whereas PKA-mediated phosphorylation is impaired by HSL phosphorylation at other sites (Kraemer et al., 2002). However, taken that also ACAT adrenal expression is unchanged across the peripartum period, it is probable that the adrenal cholesterol stores are generally depleted to fulfil high cholesterol demand. On the other hand, the SRB1 and LDLR protein expression increase at LD 8 suggests that, after several days of high basal CORT production, extra-adrenal cholesterol supply is needed.

As mentioned in the general introduction (Section 1.2.2.3), adrenal cholesterol stores have been repeatedly shown to be depleted after stress exposure and are known to be the preferential cholesterol source recruited in case of acute hormonal steroidogenesis

stimulation (Vahouny et al., 1978, Koko et al., 2004, Oliveira et al., 2009, Hu et al., 2010). Studies reported that KO of the gene encoding for HSL resulted in a 80 to 90% reduction in ACTH-induced CORT secretion from isolated adrenal cells (Kraemer et al., 2002); finding that was reproduced when cells from doubled KO mice (HSL and LDLR) were stimulated. On the other hand, KO of the LDLR gene did not influence maximal CORT production induced by ACTH (Kraemer et al., 2007). Therefore, these findings further confirm that cholesterol supply that relies on HSL activity as the selective uptake and lipid droplets recruitment play an important role for acute ACTH-induced steroidogenesis. Intriguingly, it is, therefore, highly probable that adrenal lipid droplets depletion observed in the present study during pregnancy and lactation might underlie the extensively documented reduced HPA axis stress reactivity across the peripartum period (da Costa et al., 1996, Windle et al., 1997, Neumann et al., 1998, Johnstone et al., 2000, Douglas et al., 2003).

Adrenal HMGCR protein expression was lower on LD 8 compared to V animals. This enzyme catalyzes the limiting step in cholesterol synthesis and several agents have been shown to impact on HMGCR functionality and expression, for example accelerating its degradation (Sato and Takano, 1995). Addition of LDL to the culture of UT 1 ovary cells that contain a maximal amount of HMGCR when incubated in the absence of serum suppresses HMGCR expression by 90% (Goldstein and Brown, 1977, Faust et al., 1982). The high lipoprotein-derived cholesterol influx at LD 8 might exert, therefore, an inhibitory effect on HMGCR expression, thus explaining the reduction in HMGCR protein levels that we observed at that time.

Besides the adrenal parameters involved in cholesterol delivery investigated in the present chapter, other components of the steroidogenic machinery might adapt to sustain peripartum-associated basal hyperCORT; among them the CYP450 or HSD enzymes and

StAR, protein responsible for cholesterol trafficking to the inner mitochondrial membrane (see Section 1.2.1.1). Whilst changes in StAR expression are known to impact on steroidogenesis (Lin et al., 1995), it is unknown whether such changes concur to the "peculiar" peripartum adrenocortical function. Further studies should be perform to address these issues.

As previously mentioned, OXT represents a putative candidate to trigger adrenal plasticity since OXT plasma levels are high during lactation and it has already been shown that OXT can stimulate CORT production (Stachowiak et al., 1995, Petersson et al., 1999). Specifically, single OXT subcutaneous injection (1mg/kg) was reported to cause a transient increase in CORT levels in male rats, while six hours after injection the animals showed lower CORT compared to vehicle injected rats. On the other hand, five days chronic administration decreased basal CORT for ten days after the last injection (Petersson et al., 1999). In females the effects of a single OXT injection (1µg/kg) resulted in a long lasting (12 hours) plasma CORT increase whereas ten days of daily OXT injection at two different concentrations (1µg/kg and 0.5µg/kg), treatments that provide OXT levels akin to the long-lasting increase observed during the peripartum period, decreased basal CORT without affecting ether stress-induced CORT secretion (Stachowiak et al., 1995). However, taking the high outcomes variability depending for example on the animal sex and on the administration pattern, the underlying physiological implications and OXT involvement in the regulation of corticoadrenal function is unclear, particularly during the peripartum period when the OXT system shows high plasticity. In the present study, marked changes in adrenal OXT-R binding on LD 8 were highlighted. At this time, basal HPA axis activity is still increased taken that the maternal basal hyperCORT starts to decline around LD 15 in the rat as a result of decreased suckling of the offspring that begin to ingest solid food (Voogt et al., 1969); circulating plasma OXT is also high as a result of increased pituitary secretion because of suckling stimulation

(Bruckmaier and Wellnitz, 2008). As previously mentioned, chronic high OXT levels have been shown to exert an inhibitory effect on adrenal steroidogenesis, effect that would hinder basal hyperCORT. Thus, reduction adrenal OXT-R binding might represent a strategy to avoid steroidogenesis inhibition when circulating OXT level are high during lactation. On the other hand, adrenal OXT mRNA expression differed between left and right adrenals; an effect that was independent of reproductive status. Due to the side differences in adrenal sympathetic innervations (Droste et al., 2003), changes in OXT mRNA content suggest that there may be a link . However, the measurement was limited to the mRNA expression; therefore, the observed changes in mRNA cannot be relate with changes in adrenal OXT content.

In conclusion, with the present study the adrenal glands have been shown for the first time to undergo substantial plasticity, particularly at mid-lactation. Thus, besides the extensively reported changes in brain and pituitary morphology and physiology across the peripartum period that have been proven to modulate maternal HPA axis activity, the peripartum period is characterized by profound changes in adrenal morphology and function.

Chapter 3: High-fat diet feeding and pregnancy chronic stress effects on maternal HPA axis-associated changes

3.1. Introduction

As stated in the general introduction, the profound physiological changes occurring peripartum are believed to be fundamental for the maternal mental health. Particularly regarding the HPA axis-associated peripartum plasticity, dysbalance of the HPA axis basal and stress-induced activity has been described in mothers suffering from PPD or anxiety. Indeed, women affected by PPD were reported to display an attenuated morning rise in CORT; suggesting that a subset of suffers are characterized by an hormonal profile similar to that of patients affected by post-traumatic stress disorders or atypical depression (Handwerger, 2009, Taylor et al., 2009). Even the HPA axis reactivity is impaired in mothers suffering from PPD; indeed, whereas in healthy subjects maximal treadmill exercise elicits neuroendocrine responses proportional to the intensity and duration of the exercise (Altemus et al., 1995), it has been shown to induce an imbalanced response in depressed mothers that were characterized by higher ACTH and lower CORT levels when compared to non-depressed mothers (Jolley et al., 2007).

In the last decades, several animal models have been established to study the pathophysiological mechanisms underlying postpartum mood and anxiety disorders. Specifically, chronic stress and high-fat diet based models have been reported to interfere with the HPA axis peripartum-associated plasticity and, therefore, are considered as valid tools to understand the mechanisms underlying HPA axis dysregulation and their implications for the overall pregnancy and lactation outcomes.

However, particularly regarding the high-fat diet peripartum-based models reported in the literature, the vast majority of the studies using this approach are focused on the understanding of the potential effects of the maternal diet on offspring development and health later in life. Indeed, although an interplay between obesity, including maternal obesity, and postpartum depression and anxiety, has been extensively reported (McIntyre et al., 2007, Lang and Borgwardt, 2013), the studies directly focused on the effects of high-fat diet feeding on the maternal HPA axis peripartum-associated function are limited. In line with that, the potential impacts of high-fat diet feeding on the peripartum-associated adrenal glands physiology are unknown.

In Chapter 2, several indications of peripartum-associated adrenal gland plasticity have been identified, particularly at mid-lactation in the rat and these changes are hypothesised to be necessary for the overall HPA axis function peripartum. Specifically, the importance of increased adrenal LDLR and SRB1 expression for the basal hyper-CORT at LD 8 is highly probable, when lipid droplets are substantially depleted and *de novo* cholesterol synthesis seems to be suppressed. Moreover, it is likely that adrenal lipid droplets depletion might underlie the stress hyporesponsiveness taking that cholesterol from the adrenal stores represents the preferential cholesterol source for steroidogenesis after ACTH stimuli (Hu et al., 2010).

On the other hand, a model of pregnancy chronic psychosocial stress established in our laboratory was shown to prevent basal hyperCORT in the lactating mother (Hillerer et al., 2011), finding that suggests that stress exposure might impact on adrenal peripartum physiology. However, to the best of my knowledge, pregnancy chronic stress or high-fat diet feeding effects on the adrenal glands that might underlie HPA axis dysbalance induced by these models have never been investigated.

However, whether perturbation of the fine tuned adrenal plasticity in cholesterol delivery affects the overall “peculiar” lactation-associated HPA axis (re)activity it is still unknown.

Therefore, the present study was designed to investigate whether high-fat diet feeding across the peripartum period and pregnancy chronic stress affect adrenal plasticity at mid-lactation. After showing that high-fat diet intake prevented most of the lactation-associated adrenal changes in cholesterol delivery, I further assessed whether such perturbation of adrenal plasticity might link to HPA axis dysfunction and/or to behavioural abnormalities. Therefore, the effects of high-fat diet feeding on the basal and acute stress-induced ACTH and CORT levels and on maternal care and anxiety-related behaviour were assessed.

3.2. Materials and Methods

3.2.1. Animals

Female Wistar rats (200 to 250g; Charles River, Sulzfeld, Germany) were left undisturbed for one week after arrival under standard housing (three to four rats per standard rat polycarbonate cage) and environmental (12h light-12h dark, lights on at 06:00, 22 ± 1° C, 60 ± 5% humidity) conditions. The experiments were performed between 08:00 and 11:00 unless otherwise stated. All experimental procedures were approved by the Committee on Animal Health and Care of the local government of the Oberpfalz and complied with international guidelines on ethical use of animals.

3.2.2. Experimental design

3.2.2.1. High-fat diet study

To investigate the effects of feeding a high-fat (HF) diet on maternal behavioural, physiological and molecular adaptations, three cohort of animals were exposed to the diet regimen (Fig. 23; for diet details see Section 3.2.3) starting two weeks before mating. After mating, animals were left undisturbed and grouped housed (four per cage) until PD 17 (and corresponding day in V animals) when they were single housed to allow undisturbed parturition and nurturance of the offspring. The day of parturition, offspring were reduced to eight (four females and four males per litter) and pup body weight was measured every day to monitor potential effects of maternal diet on birth weight or body weight gain until LD 7. With the first animal cohort, HF diet feeding effects on maternal and anxiety-related behaviour and on adrenal plasticity were tested. The impact of feeding a HF diet on basal and stress- (cohort two) or ACTH injection- (cohort three) induced ACTH and CORT plasma levels were assessed in the second and the third animal cohorts.

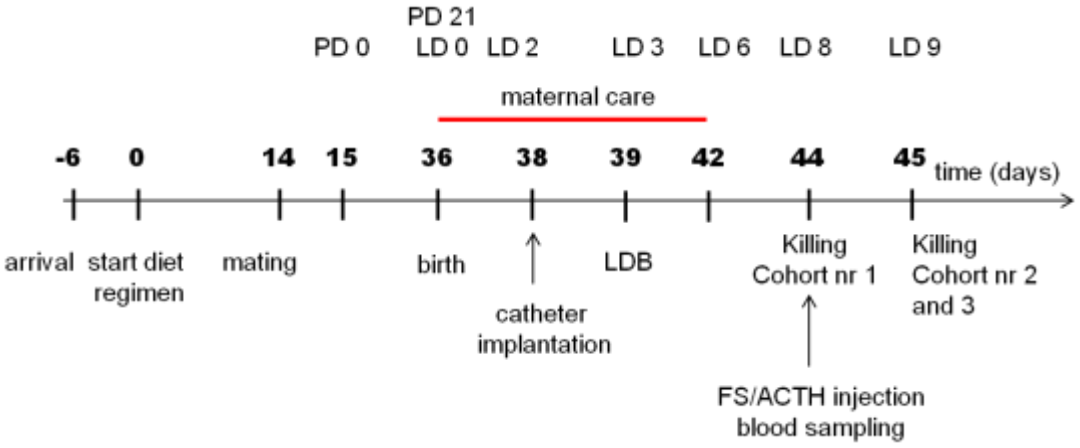


Figure 23. Schematic representation of the experimental time-line to assess the impact of high-fat diet on maternal and anxiety-related behaviour, pup parameters, adrenal plasticity and HPA axis (re)activity

One week after arrival, animals from the high-fat (HF) diet experimental group started the diet regimen while normal fed (NF) animals (the control group) continued to get access to standard rat chow. After 14 days all animals were mated until half of the females from each experimental group became pregnant and were then left undisturbed until single housing on pregnancy day (PD) 17 (and corresponding day in virgins (V)). With the first animal cohort, maternal care was observed from lactation day (LD) 1 to LD 6 with the exception of LD 3 when anxiety-related behaviour was assessed through light-dark box (LDB) testing. On LD 8 animals were killed and plasma and adrenal glands removed. The animals from the second and the third animal cohorts were implanted on LD 2 with a jugular catheter and four days later animals were exposed to an acute stressor (60 second forced swim; cohort one) or were injected intravenously with vehicle or ACTH (cohort three). Blood samples were collected before and after stress exposure/ACTH injection. Animals from the second and third animal cohorts were killed on LD 9.

N group numbers for the investigated parameters are reported in the following table.

Table 3. High-fat (HF) diet effects on maternal and anxiety-related behaviour, adrenal plasticity and HPA axis (re)activity: n numbers

		V NF	V HF	L NF	L HF
1st cohort	Body weight gain	9	8	6	7
	Adrenal weight	9	8	6	7
	ABN	-	-	6	7
	Adrenal lipid droplets	7	7	6	6
	Adrenal LDLR	5	5	6	5
	Adrenal SRB1	5	6	6	6
	Adrenal ACAT	6	6	6	6
	Adrenal HSL	6	6	6	6
	Adrenal HMGCR	6	6	6	6
	Adrenal cells CORT secretion *in vitro*	3[a]	3[a]	3[a]	3[a]
2nd cohort	Anxiety-related behaviour	8	8	7	6
	Basal and FS-induced ACTH release	7	8	7	6
	Basal and FS-induced CORT release	7	8	7	6
3rd cohort	Basal and ACTH injection-induced CORT release	5	8	7	6

a) Cells pools from six animals were incubated in triplicates

3.2.2.2. Pregnancy-stress study

I next investigated the effects of a validated pregnancy chronic stress models that has been shown to prevent maternal basal hyperCORT and to increase anxiety-like behaviour

of the dam without affecting the maternal stress hyporesponsiveness in terms of reduced CORT secretion after 60 seconds forced swimexposure (Hillerer et al., 2011). Therefore, to investigate whether this specific pregnancy chronic stress model also affects lactation-associated adrenal changes revealed in Chapter 2, the animals were mated as described in Section 2.2.3 and were exposed to the stress model described into details in Section 3.2.7 starting on PD 4 until PD 16. At the end of the stress procedure, the animals were single housed and after birth were left undisturbed until LD 8 when dams were killed and plasma and adrenal glands collected and handled as described in Sections 2.2.4 (Fig. 24). V non-stressed (NS) rats were included in the study to confirm the previously observed lactation-associated adrenal changes in order to have confidence in any stress-induced changes. However, since it has previously been published that this specific chronic stress paradigm does not affect V animals in any of the parameters assessed to date (depressive- and anxiety-related behaviour, hypothalamic AVP, CRF and OXT mRNA expression, basal and stress-induced plasma ACTH and CORT (Hillerer et al., 2011)), as was also shown in the V HF animals, the stress effects were assessed only in lactating animals.

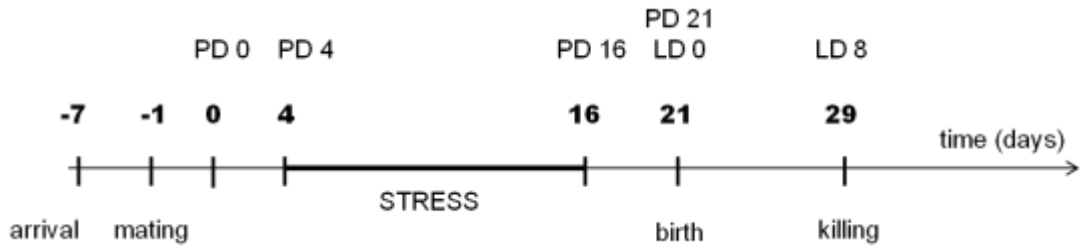


Figure 24. Pregnancy chronic stress effects on lactation-associated adrenal plasticity: experimental design

One week after arrival, animals were mated. Chronic psychosocial stress was performed between pregnancy day (PD) 4 and PD 16 while control animals were left undisturbed until single housing on PD 16. Animals were killed on lactation day (LD) 8 (or equivalent in virgin (V) animals) and adrenal glands were collected.

N group numbers for the investigated parameters are reported in the following table.

Table 4. Chronic pregnancy stress effects on peripartum plasticity: n numbers

	V NS	L NS	L S
Body weight gain	9	7	10
Adrenal weight	9	7	10
Basal plasma CORT	5	7	7
Adrenal lipid droplets	3[a]	6	6
Adrenal LDLR	6	8	8

a) Only three were required to confirm the lactation-associated decrease in lipid

3.2.3. Diet

After one week habituation, during which animals were fed with standard rat chow (10% fat; Ssniff, Germany), half of the animals from each cohort started the HF diet (45% fat; OpenSource Diets, USA) regimen. Since most of the rodent studies investigating obesity pathophysiology are performed using chow-based control diets, our control groups continued to receive the standard rat chow throughout the experiment (see (Buettner et al., 2007) for a review). Water and food were available *ad libitum.* The diet regimen was maintained throughout the all experiments.

3.2.4. Maternal care

Undisturbed maternal behaviour was assessed *via* a time sampling protocol from LD 1 to LD6 with the exception of LD 3 when anxiety-related behaviour was assessed. Between 0600 h (i.e. lights on) and 1000 h, dam's behaviour was observed for two-five s and recorded every second minute (Hillerer et al., 2011). This timeframe was chosen as it has

previously been shown to be the period most susceptible to environmental, genetic or pharmacological manipulation (Bosch and Neumann, 2008, Bosch, 2011, Hillerer et al., 2011). Behaviours addressed towards the pups like nursing, licking and grooming and pup carrying were recorded together with non pups-related behaviours like eating and drinking, resting, locomotion and digging and the behavioural display on the same day were summed. Different nursing posture were observed and particular attention was taken to distinguish between more passive nursing behaviours that do not imply a particular effort from the mother like the so called blanket posture or lying on the side nursing when the mother just lies on or closed to the pups and the more active arched back nursing (ABN). Specifically, ABN is a crouch nursing posture that implies active participation of the dam; the stretched legs and the back that delineates a characteristic arch help the observer to distinguish this posture.

3.2.5. Light-dark box (LDB) test

On LD 3 light-dark box test was performed to assess lactation and HF diet impact on anxiety-related behaviour as previously described (Slattery and Neumann, 2010). Specifically, the light-dark box consisted of a box divided into two chambers with a seven x seven cm aperture that allows the rat to freely move between the two chambers. The white floor chamber was illuminated with a ~390 lux light intensity and the dark floor chamber with ~30 lux light. The floor was marked with a ten x ten cm grid to measure locomotion activity during testing. The five minute test started by placing the animal in the middle of the light chamber facing the wall opposite to the aperture and behaviour was recorded with a video camera to leave the animal undisturbed. Behavioural outcomes to assess the anxiety level of the animals were recorded: latency first entry in the dark chamber, latency first re-entry in the light chamber, light chamber entries and rearing,

rearing in the dark chamber and total time spent in the light and dark chamber. Line-crosses in both chambers were measured to quantify locomotion.

3.2.6. Jugular catheter implantation

Animals from the second and third cohorts were implanted with a jugular catheter on LD 4 under isoflurane anaesthesia at semi-sterile conditions as formerly reported (Neumann et al., 1998, Hillerer et al., 2011). Specifically, each animal was at first shaved between the ears where the catheter will exit the body and on the chest closed to where the heart beating was visible. The chest skin was cut through a 1 cm cut specifically 5 mm far away from the heart beating. Lesion of the muscle tissue below the skin allowed the identification of the vein that was subsequently blocked through a silk string's tangle. A superficial cut of the vein allowed the insertion of a 3.3 cm silicon tube with a 0.64 mm internal diameter (SIMS Portex, UK) directed distal from the head and connected to a 13 com polyethylene catheter with a 0.58 mm diameter that was filled with heparinised saline (30 IU/ml). After placing the catheter, a second silk string was tangled to fix the junction part between silicone and polyethylene tubes with the jugular vein. The polyethylene catheter exited the body through a skin's hole between the ears and the almost five cm long catheter's part outside the body was folded to close it. The skin cut was sewn with metal stitches and disinfected with tincture of iodine whereas each animal was injected with the antibiotic Gentamicin (0.5 mg/kg). On the experimental day, when lights on, the catheter was connected to a 30 cm polyethylene tube filled with heparinised saline and terminating with a 0.6 x 25 mm needle and a 1 ml siring (Becton Dickinson, DE). Blood sampling was performed on LD 8 (all samples drawn were 0.25 ml in volume and replaced immediately with sterile 0.9% saline; Fig. 25). Specifically, two hours after lights on, and at least 90 min after attaching the catheter to the 30 cm tubing filled with heparinised saline, two basal samples were collected; plasma ACTH and CORT concentrations were

measured in the first basal sample, the second was collected as backup. Thirty minutes later, animals from the second animal cohort were subjected to 60 s forced swim in 30 cm water at 25 ± 1º C and 5, 15, 30 and 60 minutes after, blood samples were collected. Due to analytical errors one L NF was excluded from the CORT analysis whereas one V NF did not survive the surgery.

Thirty minutes after collection of the basal sample, animals from the third animal cohort received an intravenous injection through the jugular catheter of ACTH (Sigma, Germany; 0.1 ml/vol to give a final stress-related blood concentration of 500 pg/ml (Viau et al., 1993). Five, 15, 30, 60 and 120 minutes later blood samples were collected and plasma isolated as previously described (see Section 2.2.4). Plasma samples were stored at -20º C until CORT and ACTH measurement. Due to analytical errors one V NF and one HF were removed from the CORT analysis.

Figure 25. Representative picture of the experimental setting

Catheters were connected to 30 cm tubing filled with heparinized saline (30 IU/ml) two h before the beginning of any manipulation.

3.2.7. Pregnancy chronic psychosocial stress procedure

Starting four days after mating, animals were stressed *via* a chronic pregnancy psychosocial stress protocol that has been shown to impact maternal and anxiety-related behaviour and HPA axis peripartum plasticity (Hillerer et al., 2011). Two stressors were alternatively used, restraint stress (RS), starting on PD 4 (Fig. 26) and the overcrowding stress (OC), following on PD 5. Stressed animals were kept in single house cages from PD 4.

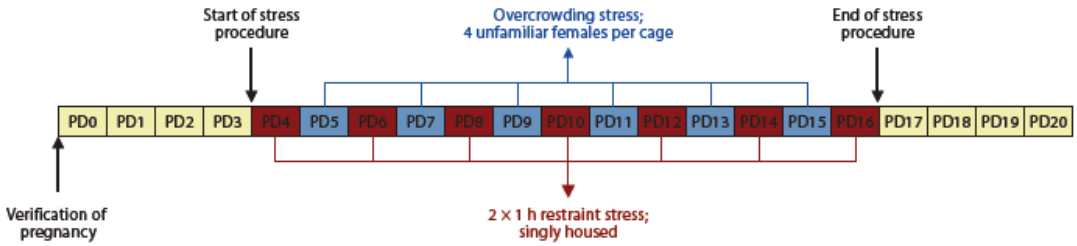


Figure 26. Schematic representation of the pregnancy chronic stress paradigm

The chronic psychosocial stress paradigm was performed between pregnancy day (PD) 4 and PD 16. Days of restraint stress (red; when the animals were restrained twice a day for 1 h) were alternated with overcrowded stress days (blue; where the four unfamiliar animals were housed in a 35 x 21 x 16 cm cage for 24 h). Dams were killed on lactation day (LD) 8 and plasma and adrenal glands collected. Adapted from (Hillerer et al., 2012)

Specifically, RS was performed twice a day, one hour in the morning (08.30-13.00) and one hour in the afternoon (13.30-17.00). The RS was performed always during the light phase and the hour varied to make the stress less predictable. Rats were placed in an acrylic glass tube of 12 cm diameter and 19 cm length, with ventilation holes.

For the OC, four unfamiliar female rats were housed together in a cage (35 x 21 x 16cm) for 24 hours. To avoid habituation that could buffer the psychosocial stress environment, the OC was performed with unfamiliar rats each time.

3.2.8. Blood and organ preparation

Trunk blood was centrifuged and plasma aliquots were stored until ACTH and CORT were assessed *via* ELISA measurement. Each adrenal, after pruning from fat was weighed. The left adrenal gland was cut in halves and one half was embedded in protective freezing medium and a series of five 16 µm cryo-sections from the mid-part of the adrenal glands was cut and then thaw-mounted onto pre-coated slides (16 µm slices containing both adrenal cortex and medulla 6 sections per slide) whereas, from the other left adrenal half, cells were isolated and incubated *in vitro* as described in Sections 2.2.4 and 2.2.9. The right adrenal gland was snap-frozen in liquid nitrogen and stored at -80° C until proteins isolation for western blot analysis.

3.2.9. Oil red lipid staining

The 16 µm cryo-sections were stained with Oil-red-O in order to assess lipids vesicles and light microscopy images of the stained adrenals were collected and analysed as described in Section 2.2.5. Briefly, the ratio between the stained area (mm^2) and the adrenal cortex area (mm^2) were measured and the stained area was expressed as percentage of the adrenal cortex (relative lipids content per cortex area).

3.2.10. Western blot analysis

Adrenal proteins were isolated *via* lysis buffer supplemented with a protease inhibitor cocktail, protein concentration was determined (see Section 2.2.8 for details) and 25µg

from each protein extract were separated *via* electrophoresis using 10% sodium dodecyl sulphate-polyacrylamide gel and transferred onto a nitrocellulose membrane. TBST at pH 7.6, supplemented with 5% milk powder, was used to block the membranes and to dilute the primary antibodies. Blots were incubated over night at 4°C with the specific primary antibody against either LDLR, SRB1, ACAT, HSL or HMGCR. For secondary antibody incubation, membranes were incubated at room temperature for one hour with peroxidase-conjugated anti-rabbit IgG in TBST. Blots were washed with TBST after each antibody incubation session. β-tubulin was used as total protein loading control. Secondary antibody incubation was performed as described above. Bands were visualised using ECL western blot detection reagents and images were acquired and analyzed (for full methodological details see Section 2.2.8).

3.2.11. ACTH-RAR

ACTH-RAR was performed using a linear ACTH-R antagonist [^{125}I]ACTH, (1-39) Tyr^{23} as tracer as described in Section 2.2.6. Briefly, the adrenal cryocut sections were thawed, dried and fixed in paraformaldehyde at room temperature. After two washing steps the slices were exposed to the tracer buffer and then washed again. Slides were then dipped in water, air-dried and exposed for 90 days to MR films. The ImageJ 1.47 program was used to measure the optical density and data were adjusted subtracting the background signal (for methodological detail see Section 2.2.6).

3.2.12. Adrenal glands cells isolation and incubation *in vitro*

The adrenal halves were homogenized DMEM/F-12, 0.1 % BSA after capsule removal and the homogenates from animals from the same experimental group were pooled. Cell homogenates were filtered and centrifuged. Supernatants were discarded and pellets re-suspended. Triplicates of 80000 cells pools from each experimental group were

incubated at 37°C. After 240 minutes the supernatant was removed and stored at -20º C until Elisa CORT measurement (for methodological details see Section 2.2.9).

3.2.13. Statistics

Results were analysed using a one-way or a two-way analysis of the variance (ANOVA) with or without repeated measures as appropriate. Any significant differences, accepted for $P<0.05$, were further analyzed using a Bonferroni *post hoc* test. If sphericity was violated P values were adjusted with Huynh-Feldt. Non parametric Mann Whitney U tests were performed when differences were not detected via ANOVA analysis. Statistical analyses were performed using IBM SPSS (version 21; Chicago, USA).

3.3. Results

3.3.1. HF diet effect on body and adrenal weight

3.3.1.1. Body weight

Two-way repeated measures ANOVA analysis showed an interaction of the diet and the day of recording on body weight gain (diet X day $F_{10,250}$=6.36; P<0.001; Fig. 27). Specifically, feeding a HF diet increased body weight gain in both V and L, effect that was significant throughout the all experiment (P values <0.05). As expected, an interaction of the factor status and day of recording was also observed (status X day, $F_{10,250}$=64.6; P0.001).

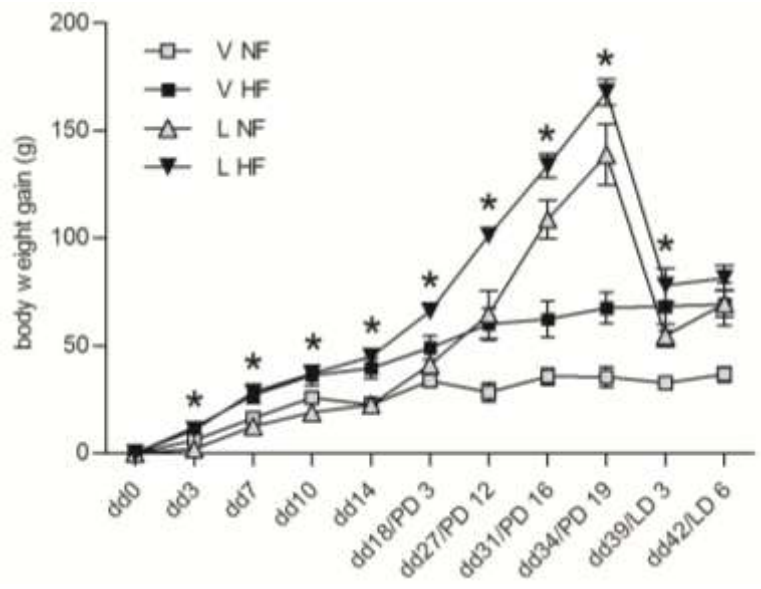


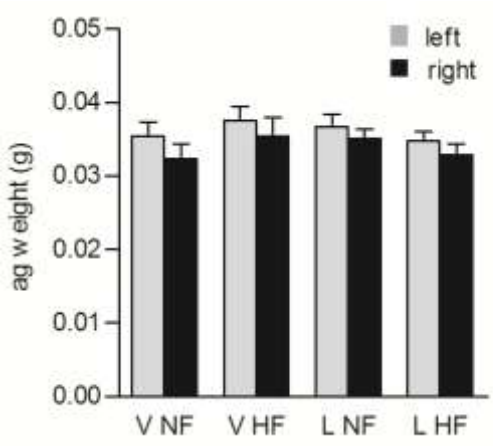
Figure 27. High-fat (HF) diet feeding increased body weight gain

Effects of feeding a HF diet on body weight gain before and after mating. Both virgins (V) and lactating animals (L) fed with a HF diet gained more weight throughout the experiment compared to normal fed (NF) rats. Data represent mean + SEM (n=6-9 per group; see tab. 3 for the exact n numbers). * P <0.05 between V NF and HF and between L NF and HF diet fed animals (Bonferroni *post-hoc*).

3.3.1.2. Absolute adrenal gland weight

Three-way ANOVA statistical analysis of the absolute adrenal glands weight reported that this parameter was nither affected by the diet nor by the status or side (Fig. 28).

Figure 28. High-fat (HF) diet feeding did not affect adrenal glands weight



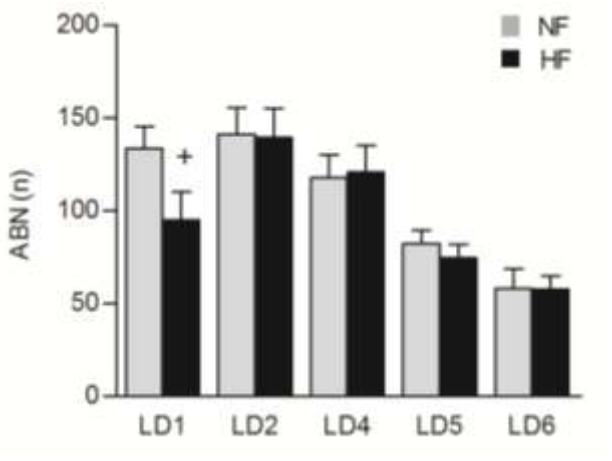
Adrenal glands weight was assessed on the day of the killing (lactation day (LD) 8 or equivalent in virgin (V) animal; e.g. after 44 days of normal chow (NF) or high-fat (HF) diet feeding) and shown not to be affected by diet, status or side. Data represent mean + SEM (n=6-7; see tab. 3 for the exact n numbers).

3.3.2. Effects of HF diet feeding on maternal, retrieval and anxiety-related behaviour

3.3.2.1. Maternal care

Two-way ANOVA repeated measures revealed a strong trend towards an interaction between diet and time on ABN behaviour (diet X day, $F_{4,36}$=2.45; P=0.064; Fig. 29). Separate statistics showed that feeding a HF diet reduced ABN on LD 1 compared to NF mothers (MWU; P<0.05).

Figure 29. High-fat (HF) diet feeding reduced arched back nursing (ABN) behaviour on lactation day (LD 1)



Nursing behaviour was scored from lactation day (LD) 1 to LD 6 to assess the effect of feeding a HF diet on this parameter. Mothers fed with a HF diet displayed less nursing behaviour on LD 1 compared to normal fed (NF) dams. Data represent mean + SEM (n=6-7; see tab. 3 for the exact n numbers). + P <0.05 vs. NF animals (Mann Whitney U).

Pup retrieval test. Diet regimen did not affect maternal retrieval behaviour (L NF: 4.9 +/- 1.2 n; L HF: 3.2 ± 0.8 n).

3.3.2.2. Anxiety-related behaviour

Lactation-associated behavioural displays of reduced anxiety were confirmed *via* two way ANOVA statistical analysis. Indeed, status affected the time when the animals re-entered the light chamber for the first time ($F_{1,25}$=5,79; P=0.024; Fig. 30 A), the number of light chamber entries ($F_{1,24}$=20.02; P<0.001; Fig. 30 B) and the rearing in the light chamber ($F_{1,25}$=5.17; P=0.032; Fig. 30 C). Specifically, NF mothers re-entered the light chamber faster (P<0.05), entered the light chamber more often (P<0.001) and showed higher rearing behaviour in light chamber (P<0.05) compared to normal fed V. These results are interpreted as reduced anxiety-related behaviour; although total time spent in the light

chamber did not differ between the groups. These adaptations were prevented by the HF diet as behavioural outcomes did not differ between V and L HF diet animals nor V NF and V HF; although no statistical differences were observed between L NF and L HF groups. Rearing behaviour and locomotion (line-crosses) did not differ between the groups (data not shown).

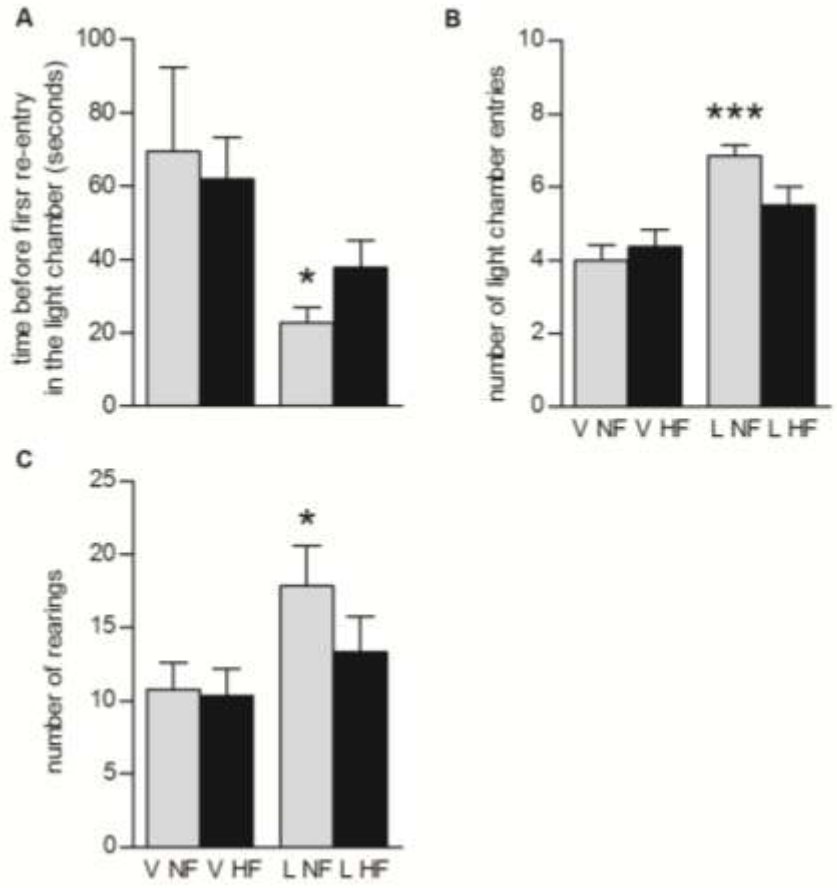


Figure 30. High-fat (HF) diet prevented lactation-associated anxiolysis

Figure legend: Light-dark box testing was performed to investigate the impact of feeding a HF diet on anxiety-related behaviour. Normal fed (NF) mothers needed less time to first re-enter the light chamber (A), entered the light chamber more often (B) and displayed higher rearing behaviour in the light chamber (C) compared to NF virgins (V). Such differences were prevented by HF diet feeding although the behaviour of L HF mothers did not statistically differ from L NF. Data represent mean + SEM (n=6-8; see tab. 3 for the exact n numbers). * * * P<0.001, * P<0.05 vs. V NF (Bonferroni *post-hoc*).

3.3.3. Effects of HF diet feeding on adrenal-related parameters (Cohort 1)

3.3.3.1. Adrenal ACTH-RAR and lipid droplets

Two-way ANOVA reported that neither status nor diet affected adrenal ACTH-RAR (V NF: 12.22 ± 2.4 OD; V HF: 11.75 ± 3.4 OD; L NF: 10.03 ± 2.4 OD; L HF: 10.97 ± 2.8 OD).

An interaction between status and diet on adrenal cortical lipid droplets was observed ($F_{3,25}$=19.9; P<0.001; Fig. 31 A). Adrenal droplets depletion on LD 8 compared to V was demonstrated in NF animals (P<0.001); confirming my results obtained in Chapter 2. NF mothers stores were significantly depleted even compared to HF mothers (P<0.001) while lipid droplets were decreased in HF diet dams compared to V (P<0.05).

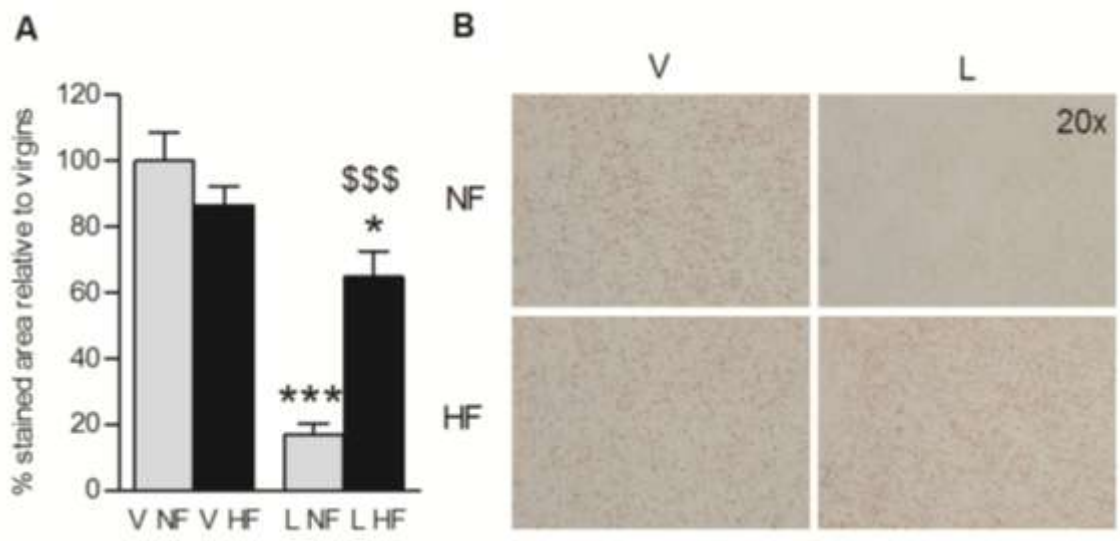


Figure 31. High-fat (HF) diet feeding prevented the lactation-associated depletion of cortical lipid droplets

The oil-red stained lipid droplets area *per* adrenal cortex area in six adrenal sections for each animal. Adrenal lipid droplets were markedly depleted in lactating normal fed (LNF) animals compared to virgins (V) NF. Feeding a HF diet partially prevented this adaptation (A). Data represent mean + SEM (n=6-7; see tab. 3 for the exact n numbers). * * * P<0.001 and * P<0.05 compared with respective virgin control group; $$$ P<0.001 vs. lactating NF dams (Bonferroni *post-hoc*). Representative pictures of stained adrenal sections are shown (20x magnification; B).

3.3.3.2. Adrenal protein expression

Two-way ANOVA revealed that both status ($F_{3,20}$=26.4; P<0.001) and diet ($F_{3,20}$=16.4; P=0.001) affected adrenal LDLR expression (Fig. 32 A). *Post-hoc* analyses revealed a lactation-induced increase in LDLR compared to V (NF animals P<0.001; HF diet animals P<0.05); replicating the results obtained in the previous Chapter. Compared to NF dams, HF L animals showed a decrease in LDLR adrenal expression (P≤0.001). Diet manipulation did not affect SRB1 expression whereas the status effect on adrenal SRB1 that was revealed in Chapter 2 was confirmed ($F_{3,22}$=69.6; P<0.001; Fig. 32 B); SRB1 adrenal protein levels were increased in both NF and HF L animals compared to the respective V groups (P<0.001). Neither status nor diet affected ACAT or HSL adrenal expression (Fig. 32 C and 32 D, respectively). The status effect on HMGCR expression reported in Chapter 2 was also confirmed ($F_{3,23}$=5.26; P=0.002; Fig. 32 E) with the enzyme being lower on LD 8 compared to V in NF rats (P<0.01); this status effect was prevented in the HF diet group.

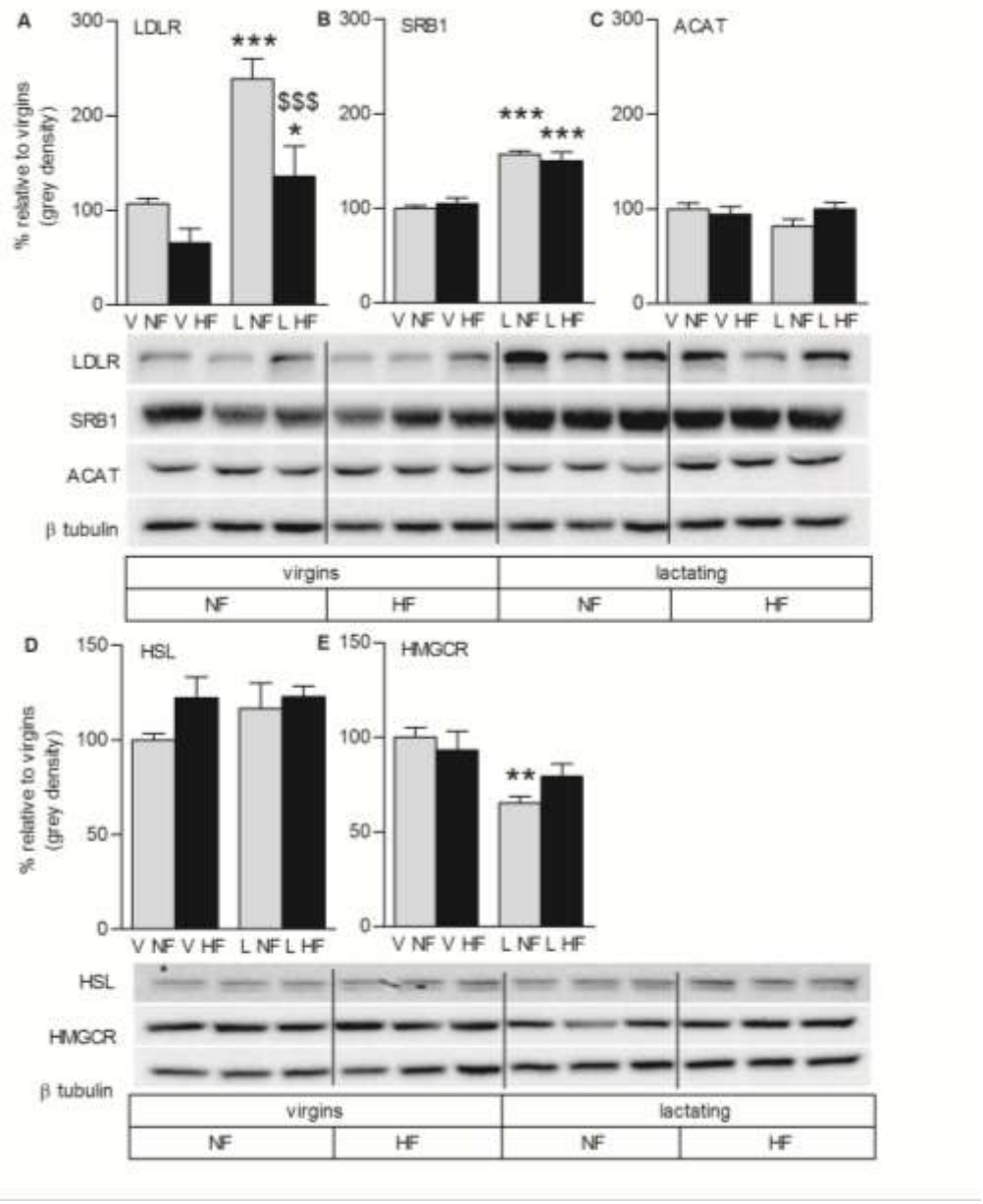


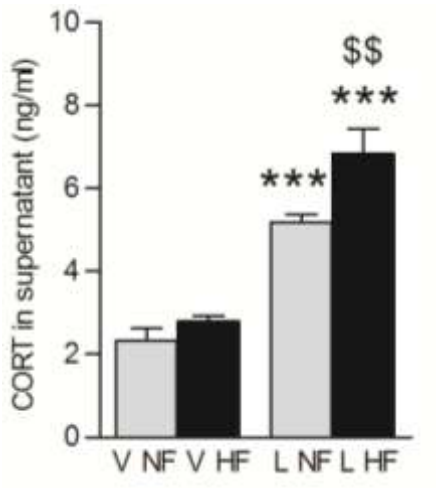
Figure 32. High-fat (HF) diet feeding partially prevented the lactation-associated changes in adrenal expression of proteins involved in cholesterol delivery

Figure legend: Western blot analysis was performed to assess the impact of feeding a HF diet on adrenal protein expression. Feeding a HF diet prevented the lactation-induced changes in LDLR and HMGCR expression without affecting the lactation-associated increase in SRB1. Data represent mean + SEM (n=5-6 per group; see Tab. 3 for the exact n numbers) from western blot analysis of adrenal LDLR (A), SRB1 (B), ACAT (C) HSL (D) HMGCR (E), which were normalized to the loading control, β-tubulin, in virgins (V) and lactating (L) NF and HF diet animals. * * * P<0.001, * * P<0.01, * P<0.05 vs. respective V control group. $ $ $ P≤0.001 vs. L NF dams (Bonferroni *post-hoc*). Representative blots of the proteins of interest and the loading control are reported below the graphs.

3.3.3.3. Adrenal cells incubation *in vitro*

The status effect on the *in vitro* CORT production from isolated adrenal cells showed in Chapter 2 was confirmed with the present study ($F_{3,11}$=99.9; P<0.001; Fig. 33). Moreover, CORT release was also affected by the factor diet ($F_{3,11}$=9.58; P=0.015). Adrenal cells from L animals secreted higher amount of CORT compared to the respective V group (P<0.001) in both NF and HF diet fed animals. Furthermore, CORT secretion differed between HF and NF dams with HF mothers showing grater secretion than NF dams (P<0.01).

Figure 33. High-fat (HF) diet feeding further increase the *in vitro* CORT release from isolated adrenal cells from lactating animals (L) compared to virgins (V)



Adrenal cells pools from V and lactating L normal fed (NF) and high-fat (HF) diet animals after isolation were incubated *in vitro* for 4h. Cells pooled from L animals secreted more CORT compared with cells pools from V. Cells from HF diet mothers showed an even greater CORT production compared to L NF. Data represent mean (in triplicate) + SEM (n=3). * * * P<0.001 vs. respective V control group. $ $ P≤0.01 vs. L NF (Bonferroni *post-hoc*).

3.3.4. Effects of HF diet feeding on HPA axis (re)activity

3.3.4.1. Plasma ACTH and CORT before and after acute stress exposure (Cohort 2)

Two-way ANOVA repeated measures analysis showed that basal and FS stress induced ACTH levels were affected by time ($F_{2,20}$=36.8; P<0.001; Fig. 34 A). Plasma ACTH levels rose in all groups after stress exposure and return to basal level 60 min after stress termination. The increase in plasma ACTH in L mothers compared to V that has been reported in Section 2.3.2.1 was confirmed with the present study in NF animals through separate statistical analysis of the basal plasma ACTH levels (Mann Whitney U; P≤0.001; Fig 34 B). Feeding a HF diet prevented this adaptation.

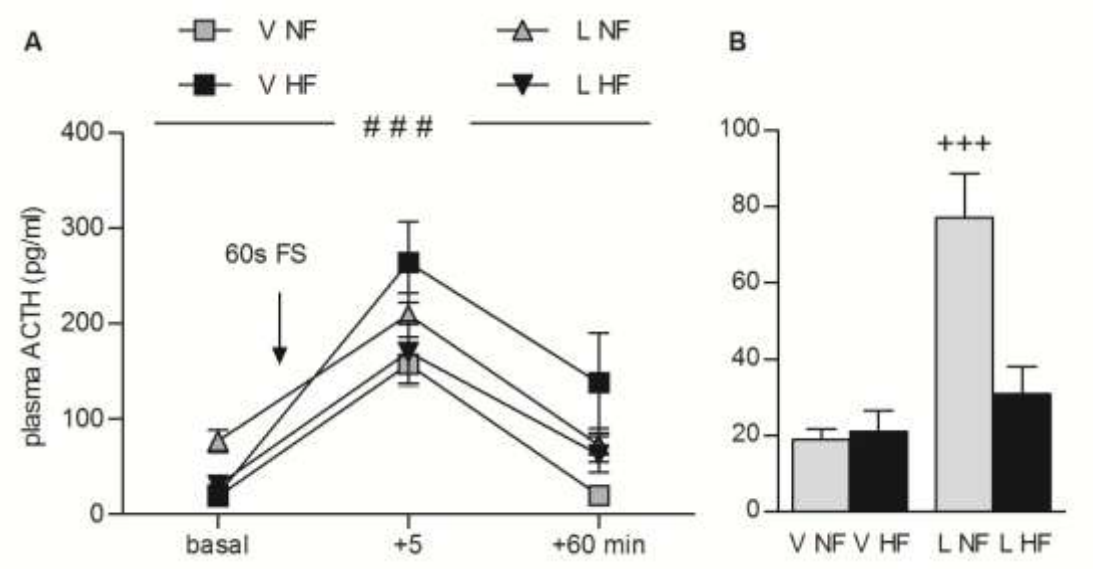


Figure 34. High-fat (HF) diet feeding prevented the lactation-associated increase in basal plasma ACTH

Basal and 60 seconds (s) forced swim (FS) stress induced plasma ACTH levels in virgin (V) and lactating (L) animals fed on a HF diet or normal chow (NF) is represented (A). ACTH increased after stress exposure in all experimental groups. Feeding a HF diet prevented the lactation-associated basal high ACTH levels (B). Data represent mean + SEM (n=6-7; see tab. 3 for the

exact n numbers). # # # P<0.001 main ANOVA effect of time; + + + P≤0.001 L NF vs. V NF after Mann Whitney U test analysis.

Two-way repeated measures ANOVA statistical analysis reported a main interaction effect of time and status on CORT release (time X status; $F_{4,76}$=4.16; P=0.008; Fig. 35 A). CORT response to 60 seconds FS was attenuated in both NF and HF L animals compared to the respective V groups 5, 15 and 30 minutes after stress terminaltion (P<0.05). Basal hyper-CORT was confirmed in NF dams compared to V (P<0.01), lactation-associated change that was abolished in the HF diet group (Fig. 35 B). Two-way ANOVA analysis of the CORT area under the curve (AUC) measured from the baseline of each group confirmed the attenuated CORT response to stress exposure in L rats ($F_{3,24}$=34.4; P<0.001; Fig. 35 C). Indeed, both NF and HF L were characterized by a lower CORT AUC compared to the respective V control group (P<0.001).

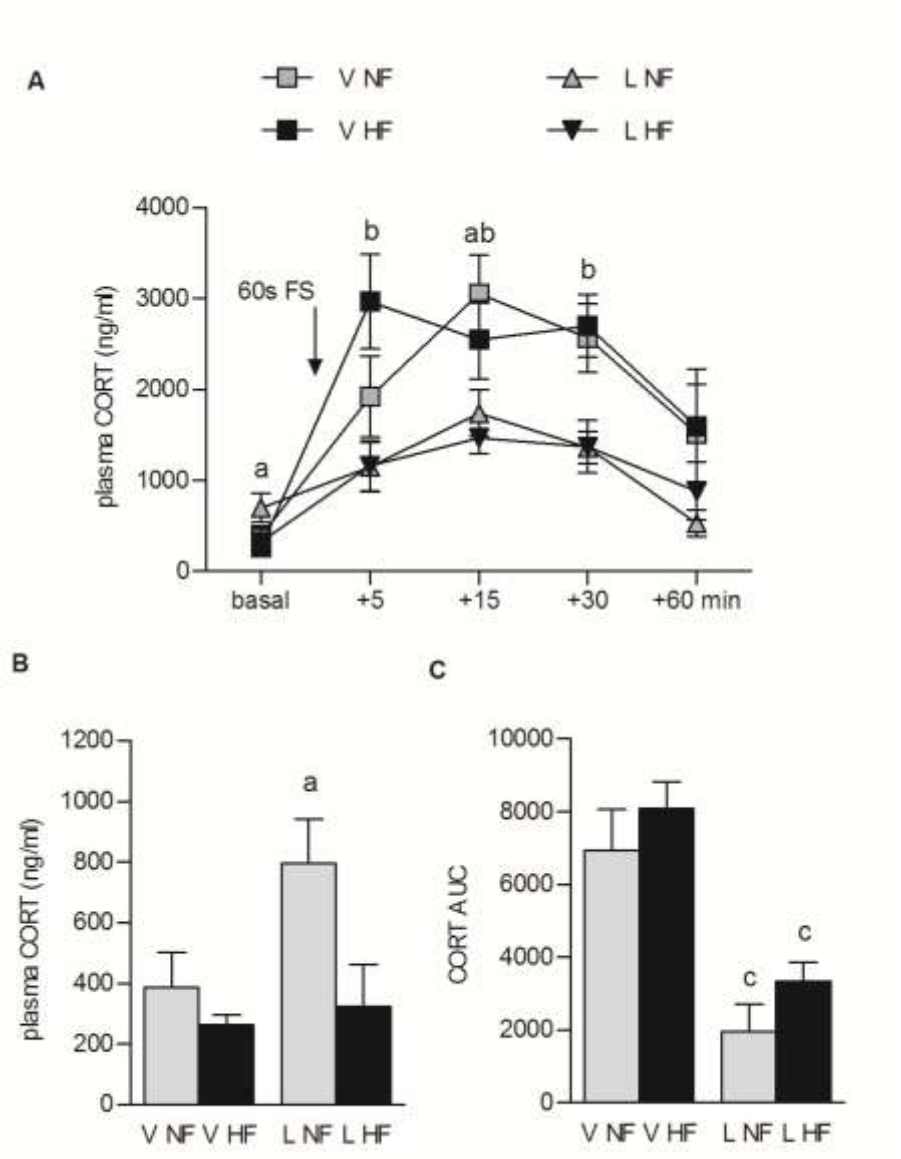


Figure 35. High-fat (HF) diet feeding affected basal but not stress-induced CORT levels in lactating (L) animals

Basal and 60 seconds (s) forced swim (FS) stress-induced plasma CORT levels in virgin (V) and lactating (L) animals fed on a HF diet or normal chow (NF) are represented (A). L animals displayed a lower CORT response to acute stress exposure compared to V rats (A) and this finding was confirmed *via* CORT area under the curve (AUC) measured from the baseline of each group (C). In panel B, a separate representation of the HF diet-induced prevention of the basal hyper-CORT is provided. Data represent mean + SEM (n=6-7 per group; see tab. 3 for the exact n numbers). a $P<0.01$ between V and L NF; b $P<0.05$ between V and L HF, c $P<0.001$ vs. the respective V group (Bonferroni *post-hoc*).

3.3.4.2. Plasma CORT before and after ACTH intravenous injection (Cohort 3)

Two-way repeated measures ANOVA statistical analysis revealed that ACTH injection-induced CORT secretion was affected by the time and the diet (factor time X diet; $F_{5,65}$=0.921; P=0.013; Fig. 36 A) and by the status ($F_{1,13}$ =10.5; P=0.006). The basal hyper-CORT was confirmed in NF dams compared with V NF (P<0.05; Fig 36 A and B) and it was prevented by HF diet confirming the findings from Cohort 2. HF mothers displayed an exaggerated CORT response 5 and 15 minutes after ACTH injection compared to L NF (P<0.01 and P<0.05, respectively) and compared to V HF 5 minutes after the injection (P<0.01) but no differences at other time points were observed. Assessment of the CORT AUC measured from the baseline of each group confirmed the lactation-associated hypo-response in terms of CORT secretion in L NF animals compared to V; the CORT response was fully restored in the L HF diet group. Indeed, statistical analysis showed that status and diet interact (factor status X diet; $F_{1,24}$=6.79; P=0.017; Fig. 36 C) thus preventing the reduced CORT production after ACTH injection in lactating dams (L vs. V NF P<0.05). The CORT AUC was significantly higher in L HF compared to LNF (P≤0.001).

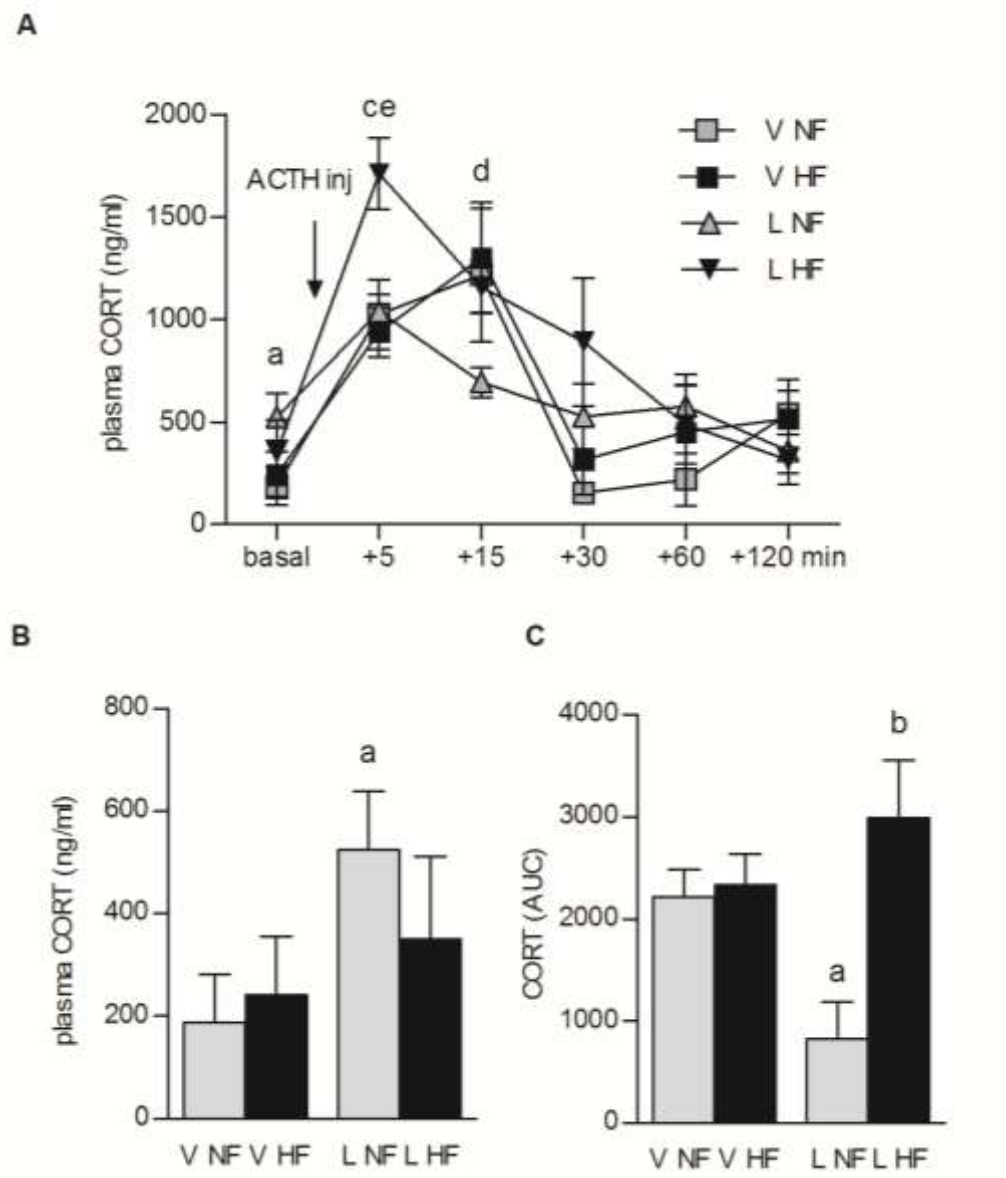


Figure 36. High-fat (HF) diet exaggerated CORT response to ACTH intravenous injection in lactating (L) animals

Basal and intravenous ACTH injection (inj)-induced plasma CORT levels in virgin (V) and L animals fed on a HF diet or normal chow (NF) are represented (A). In L animals, feeding a HF diet prevented the basal hyper-CORT and exaggerated the plasma CORT increase after ACTH injection (A, B, C). CORT area under the curve (AUC) measured from the baseline of each group confirmed that feeding a HF diet prevented the hypo-response to ACTH injection observed in L NF animals compared with V NF (C). P<0.01 between V and L NF; b P<0.001, c P<0.01 and d P<0.05 between L NF and L HF; e P<0.01 between V HF and L NF (Bonferroni *post-hoc*). Data represent mean + SEM (n=5-8 per group; see tab. 3 for the exact n numbers).

ACTH plasma levels were measured to confirm the lactation-associated changes in basal ACTH that were prevented by feeding a HF diet in the previously described cohort 2 experiment and to monitor the ACTH concentrations reached after injection. The previously mentioned lactation-induced increase in basal plasma ACTH was confirmed and also the HF diet effect on this parameter. Specifically, separate statistical analysis revealed that basal plasma ACTH prior to the injection was higher in L NF animals compared to V (V NF 22.48; L NF 78.53 pg/ml; Mann Whitney U $P<0.01$; data not shown). Feeding a HF diet prevented this lactation-associated adaptation (V HF 20.02; L HF 41.81 pg/ml; Mann Whitney U $P=0.081$). Five minutes after injection, plasma ACTH was higher in all groups (V NF 346.7; V HF 253.7; L NF 248.6; L HF 311.66 pg/ml) compared to basal and progressively decreased to rich basal concentration 120 minutes after the injection in all groups (V NF 36.14; V HF 48.35; L NF 37.69; L HF 32.18 pg/ml).

3.3.5. Chronic stress effects on body and adrenal weight

3.3.5.1. Body weight

An interaction effect of stress and time was observed *via* one-way repeated measures statistical analysis of the body weight gain throughout the peripartum period ($F_{27,405}=4.44$; $P=0.016$; Fig. 37). S animals showed lower body weight gain compared with NS animals (P values <0.05).

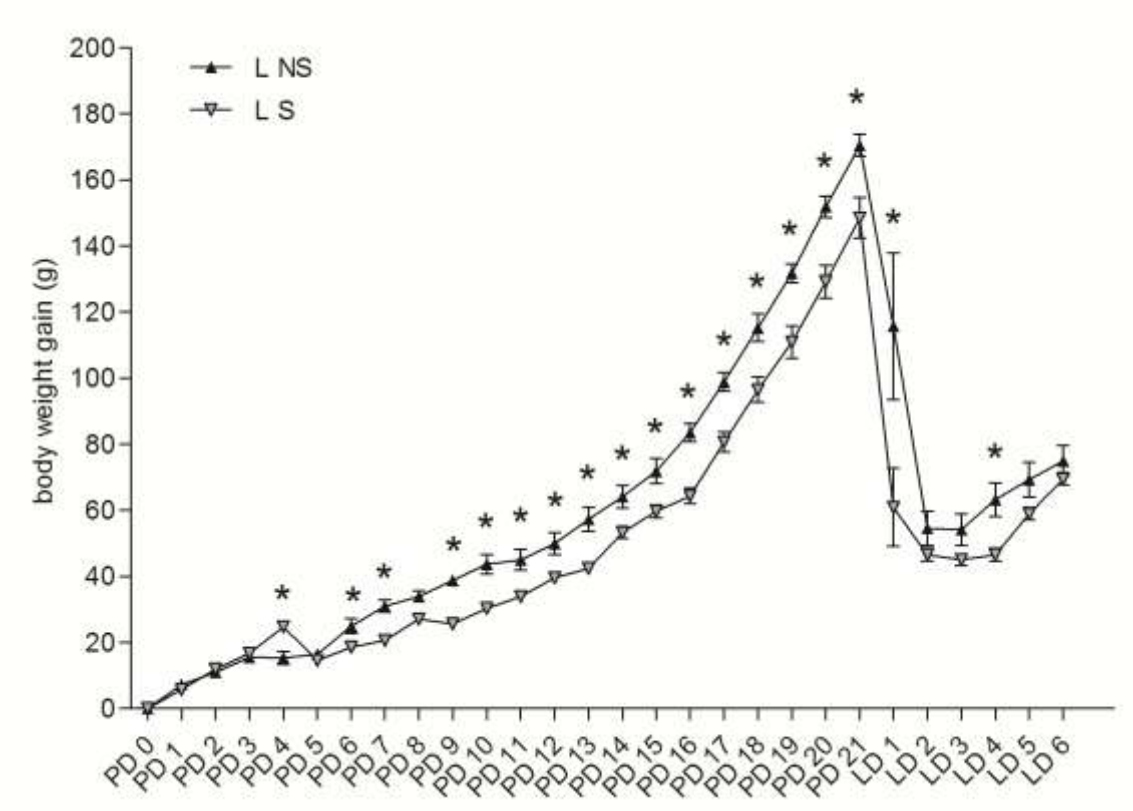


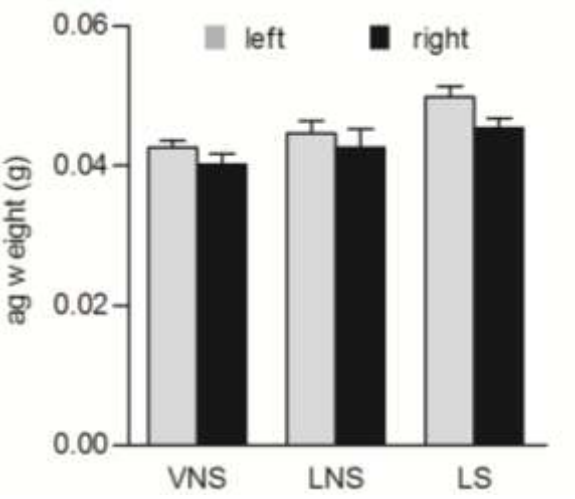
Figure 37. Chronic stress reduced body weight gain throughout the peripartum period

Exposure to chronic psychosocial stress (from pregnancy day (PD) 4 to PD 16) led to a decreased body weight gain throughout pregnancy and lactation. Data represent mean + SEM (n=7-10; see tab. 4 for the exact n numbers). * P <0.05 compared with NS animals (Bonferroni *post-hoc*).

3.3.5.2. Absolute adrenal glands weight

Two-way ANOVA statistical anlysis was performed to assess the effects of status and side between V and L NS animals and the effects of side and stress between L NS and S on the absolute adrenal glands weight. Neither status nor stress or side affected this parameter (Fig. 38).

Figure 38. Adrenal glands weight did not differ across the experimental groups



Exposure to chronic psychosocial stress (from pregnancy day (PD) 4 to PD 16) did not affect absolute adrenal glands weight recorded the day of the killing (lactation day (LD) 8 or equivalent in virgin (V) animal). Moreover, this parameter was neither affected by the animal status (virgin (V) vs. lactating (L)) nor by the adrenal side. Data represent mean + SEM (n=7-10; see tab. 4 for the exact n numbers).

3.3.6. Chronic stress effects on adrenal parameters

3.3.6.1. Adrenal lipid droplets

Student's t test analysis confirmed that adrenal cortical lipid droplets were depleted in L NS dams compared to V (t_9=2.59; P<0.05; Fig. 37 A), further confirming the findings from Chapter 2. Exposure to chronic pregnancy stress did not affect this strong lactation-associated adaptation.

3.3.6.2. Adrenal protein expression

Student's t test statistical analysis confirmed that adrenal LDLR expression was increased in L NS animals compared to V (t_{12}=4.88; P<0.001; Fig 37 B) as revealed in Chapter 2. Chronic pregnancy stress did not affect the lactation-associated LDLR increase.

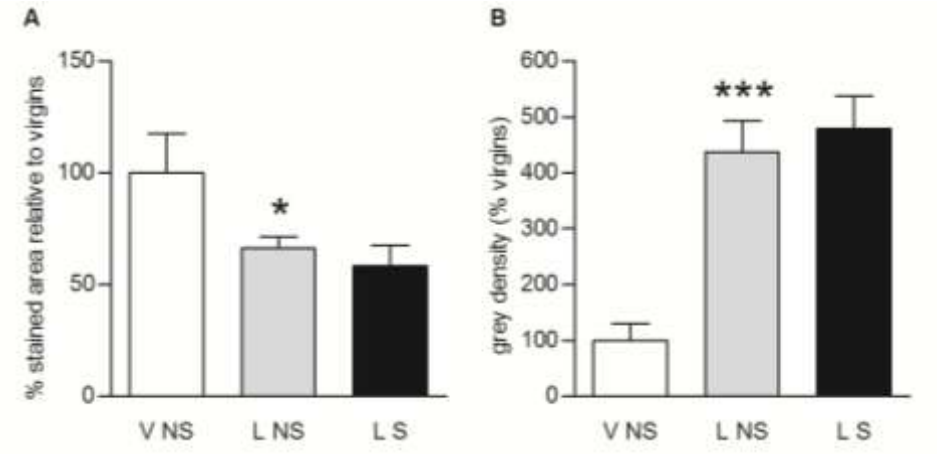


Figure 37. Pregnancy chronic stress did not affect the lactation-associated changes in adrenal lipid droplets and LDLR expression

The lipid droplets area per cortex area in oil-red stained adrenal sections is represented (A). Lactation-induced reduction in adrenal lipid droplets was not affected by pregnancy chronic stress. Even the lactation-associated increase in adrenal LDLR expression was not affected by the stress (B). Data represent mean + SEM (n=3-8; see tab. 4 for the exact n numbers). * * * P<0.001 and * P<0.05 compared to V NS.

3.4. Discussion

The present study was designed to examine the effects of two animal models (diet induced obesity and chronic pregnancy stress) on lactation-associated HPA axis plasticity and behaviour. I could demonstrate that exposure to a HF diet throughout the peripartum period decreased nursing behaviour on LD 1, increased anxiety-like behaviour in lactating rats and caused substantial alterations in lactation-associated HPA axis plasticity. In more detail, HF diet exposure prevented many lactation-associated adrenal adaptations, as well as basal hyper-CORT and ACTH, but not FS-induced changes in plasma CORT levels. In contrast, while chronic stress abolishes the lactation-associated basal hyper-CORT, I did not observe alterations in the main adrenal parameters. Taken together, these results suggest that different mechanisms underlie the basal CORT effects of HF and chronic stress.

Verification of successful implementation of HF diet

In order to confirm the effectiveness of HF diet feeding, body weight was recorded throughout the duration of each experiment. I could show that HF diet fed animals showed increased body weight gain starting three days after diet regimen began until LD 6 (and corresponding day in V) when body weight was recorded for the last time. This increase in body weight gain in HF diet animals is in line with most of the findings reported in the literature that show that prolonged feeding with diet enriched in fat normally induces a body weight increase that ranges between 10 and 20 % compared to animals fed with standard rat chow (see (Buettner et al., 2007) for review). However, it is important to consider that metabolic indications of obesity like alterations in body weight gain are highly dependent on the experimental design in terms of animal strain studied or fat

percentage in the diet and duration of the diet exposure. For example, two studies performed in female rats reported no difference in body weight gain when Sprague-Dawley rats were fed with a 28 % fat diet throughout pregnancy and lactation starting eight weeks before mating (Franco et al., 2012) or when Wistar female rats were fed with a 60 % fat starting at the beginning of pregnancy (Purcell et al., 2011). Indeed, the elevation in body weight observed throughout the experiments of the present study was due to the initial increase observed before mating. On the other hand, male mice that were exposed to a 45% fat diet were shown to develop obesity and insulin resistance with increased body weight gain already manifested within the first week of diet regimen compared to control animals (Auvinen et al., 2012).

HF effects on maternal behaviour and anxiety

Maternal care observation revealed reduced ABN behaviour the day after parturition in HF diet mothers compared with controls. Reduction in ABN may result from a variety of HF diet-induced deficits in maternal motivation and physiology. For example, HF diet feeding has already been shown to delay lactogenesis in mice (Flint et al., 2005) and pre-pregnancy overweight and obesity were associated with diminished PRL response to suckling in rats (Rasmussen and Kjolhede, 2004). In line with the results from the previously mentioned rodent studies, low rates of lactation initiation has been extensively associated with maternal overweight and obesity in women (Donath and Amir, 2000, Hilson et al., 2004). Thus, reduced ABN the day after parturition might underlie a delay in lactogenesis or diminished PRL response to suckling. However, since ABN normalized on LD 2 , the former explanation appears more likely; although future experiments would be necessary to verify this. As mentioned in the general introduction (see Section 1.5.5), a variety of repeated stress paradigm, as well as exogenous CORT exposure, have been

shown to affect maternal care (Maestripieri et al., 1991, Pardon et al., 2000, Smith et al., 2004, Brummelte and Galea, 2010, Hillerer et al., 2011, Nephew and Bridges, 2011, Murgatroyd and Nephew, 2013). Deficits in maternal behaviour have been extensively reported to impact on offspring development and health later in life (Szyf et al., 2005, Ivy et al., 2008, Starr-Phillips and Beery, 2013) and are also associated with postpartum stress-related disorders such as anxiety and mood disorders (Perani and Slattery, 2014). Specifically, maternal stress and obesity have been shown to exert profound effects on the offspring physiology and behaviour later in life. Offspring from stressed or obese mothers, for example, show higher anxiety-like behaviour and hyperactive HPA axis in adulthood (Brunton, 2010, Sasaki et al., 2013) alterations that increase their likelihood of developing somatic and psychiatric disorders.

Light-dark box testing confirmed the known anxiolytic-like phenotype of NF mothers compared to V NF. Specifically, NF mothers showed lower latency to re-enter the light box and an increased rearing in the light chamber; typically considered measures of anxiety-related behaviour (Cryan et al., 2003) Interestingly, HF diet mothers did not differ from the respective V control group in any of the previously mentioned parameter thus indicating that feeding a HF diet prevented the lactation-associated anxiolysis. Taken that the test started placing the animals in the light box, it is not surprising that the total time spent in the light box did not differ between the groups. Indeed, with this experimental setting, it is not possible to exclude that anxious animals do not move to the dark chamber because afraid of environmental changes. However, it would be necessary to confirm these results with further tests for anxiety-related behaviour, such the elevated plus maze test. The increased anxiety-like behaviour observed in HF dams mirrors results from human studies reporting elevated anxiety- at 4-months, and depressive symptoms at both 4- and 14-months, postpartum in overweight women. Moreover, in the same study, 4-month BMI predicted 14-month postpartum depressive symptoms (Carter et al., 2000).

Another human study showed that in obese pregnant women the levels of state anxiety significantly increased from trimester one to trimester three, while this parameter remained constant throughout pregnancy in normal-weight women (Bogaerts et al., 2013). Therefore, it would be interesting to determine if the HF diet used in my studies alters anxiety- or depressive-related behaviour in pregnancy as well, which could be assessed in future studies.

HF diet effects on adrenal morphology and function

Despite the previously mentioned diet marked effects on body weight, feeding a HF diet did not affect absolute adrenal gland weight. This parameter also did not differ between V and L animals, thus confirming the finding from the previous chapter showing that the reproductive status does not affect adrenal glands mass.

As an important pre-requisite, with the present study I could recapitulate the lactation-associated adrenal changes in cholesterol delivery pathways described in the previous Chapter in this thesis. This recapitulation serves not only to validate my initial findings, but reveals that these peripartum-associated adrenal changes are robust adaptations and increased the significance and importance of the observed HF diet effects on these parameters. Specifically, lipid droplets, which were again shown to be highly depleted on LD 8 in NF mothers, were restored at V levels by feeding a HF diet. This effect was lactation-specific since there was a tendency for the diet to actually decrease lipids in V animals. Taken the hypothesised involvement of adrenal lipids depletion for the maternal stress hyporesponsiveness, hypothesis that has been discussed in the previous Chapter (see Section 2.4), the observation that feeding a HF diet prevented adrenal lipids depletion strongly suggests that the maternal HPA axis *in vivo* function might also be

affected. To assess this issue, *in vivo* analysis of the HPA axis (re)activity in catheterized animals was performed; the related findings are discussed in the following discussion section. Adrenal HMGCR protein expression that was shown in the previous chapter to be decreased on LD 8 was normalized to V levels by HF diet feeding. On the other hand, adrenal ACTH-R binding, parameter that was shown in the previous chapter not to vary across the peripartum period, was also not affected by HF diet feeding, neither in V nor in L rats. HF diet intake partially prevented the lactation-associated increase in LDLR expression although the reason for the LDLR increase not being completely abolished was that feeding a HF diet tended to decrease its expression in V animals. The findings from the previous chapter and from studies performed in LDLR and HSL KO mice suggest that LDLR adrenal increase on LD 8 is important to sustain maternal basal hyperCORT ((Kraemer et al., 2007) see Section 2.4). This hypothesis is further supported by the present finding taking that the HF diet induced effect on LDLR expression was accompanied by prevention of the maternal basal hyperCORT *in vivo.* On the other hand, the lactation-induced increase in SRB1 expression shown in the previous chapter was not affected by HF diet feeding; the receptor levels were high in L HF compared to V as in NF animals. Interestingly, enhanced cholesterol availability due to increased adrenal SRB1 expression has been hypothesised to underlie the CORT hyper-response of chronic stressed mice to heterotypic stressor exposure *in vivo* (Uschold-Schmidt et al., 2012, Fuchsl et al., 2013). Thus, the increased SRB1 levels in L HF mothers whose adrenal cholesterol stores in place implies an excess in cholesterol availability for steroidogenesis, situation that could link to dysregulation of the *in vivo* CORT levels as reported for the stressed male mice mentioned above.

HF diet effect on lactation-associated *in vivo* HPA axis activity

Given such wide-ranging effects on adrenal plasticity, and their implications for basal and stress-induced CORT levels, I next assessed whether HF diet feeding alters lactation-associated *in vivo* HPA axis function. I could show that basal HPA axis activity in lactating animals was markedly affected by the diet. Specifically, feeding a HF diet prevented the lactation-associated increase in basal plasma ACTH and CORT levels (Stern et al., 1973, Walker et al., 1992, Shanks et al., 1999, Heinrichs et al., 2001, Lightman et al., 2001, Hillerer et al., 2011). Despite these marked HF diet-induced alterations in the *in vivo* HPA axis basal activity, the hyperCORT production from isolated adrenal cells *in vitro* reported in the previous chapter was not affected. Specifically, adrenal cells from both, NF and HF lactating rats, produced significantly higher CORT compared with cells from V animals; moreover, the adrenal cells CORT production was significantly higher in L HF compared to L NF. This discrepancy between *in vivo* and *in vitro* outcomes suggests that HF diet-induced adrenal changes potentiate CORT production in the absence of *in vivo* factors that might buffer such effects; such as the adrenal sympathetic innervation and OXT, two important systems that show profound plasticity peripartum and that are known to influence adrenal steroidogenesis. Future studies are needed to clarify these hypothesis taken that OXT has already been shown to directly impact on adrenal *in vitro* CORT release from isolated adrenal cells. Specifically, it has been shown that OXT enhanced basal CORT production from isolated adrenal cells and depressed the CORT response to supraphysiological ACTH doses. In the same study, OXT was shown to increase both, basal and ACTH induced CORT secretion from adrenal cells from rats that were chronically injected (10 days) with OXT *in vivo*, situation that mimic the lactation-associated increase in circulating OXT (Stachowiak et al., 1995). However, the changes induced by HF diet feeding on the OXT system peripartum and on the implications of such changes for the HPA axis function are still poorly understood.

I could next show that HF diet prevented the CORT hyporesponse to intravenous ACTH injection at stress concentrations in L HF. This approach was chosen to highlight whether HF diet interfering with adrenal peripartum plasticity directly impacts on the *in vivo* CORT production. Therefore, taken together with the findings regarding the adrenal cholesterol esters stores availability and the increased SRB1 expression, the hyperresponse to ACTH injection supports the hypothesis that increased cholesterol availability increase CORT response to ACTH stimulation and, vice versa, lipid droplets depletion might participate to the stress hyporesponsiveness in NF mothers. This result is particularly interesting given that obese women were shown to hyper-respond to ACTH intravenous injection (Marin et al., 1992), a finding that confirms this specific diet-induced obesity model as a suitable translational method to investigate HPA axis involvement in the pathophysiology of obesity. However, it is not known whether investigation of the effect of intravenous ACTH injection in obese women peripartum would result in the same effect. In line with that, in the present study, feeding a HF diet did not affect HPA axis associated-morphology and function in V animals whereas the effects in the study from Marin and colleagues were collected in non pregnant women. Taken together these findings suggest that the peripartum-associated physiology is particularly susceptible to environmental changes and, therefore, stronger manipulations are needed to exacerbate maladaptive effect of feeding a HF diet in V animals such as exposure to higher fat percentage or for a longer time.

Despite basal and ACTH-induced HPA activity was markedly affected by the diet, this was not the case for the reactivity to acute stress exposure. Indeed, neither ACTH nor CORT plasma levels after 60 s forced swim were altered in the HF diet groups compared to NF animals. As expected, CORT levels were reduced in L dams compared with V. However, this marked reproductive status-related difference in plasma CORT was not paralleled by changes in ACTH after stress exposure. Indeed, plasma ACTH increased after stress to

the same extent in L dams as in V animals; a finding that supports the hypothesis of a prominent adrenal glands role in the mediation of stress hypo-responsiveness as already speculated (Reber, 2012)by others (Hillerer et al., 2011, Reber, 2012). However, it is important to mention that the peripartum stress-induced ACTH release pattern is still unclear taken that studies reporting both, equal ACTH release in V and L rats (Hillerer et al., 2011) and decreased ACTH in L dams (Neumann et al., 1998, Shanks et al., 1999) after stress exposure are reported in the literature. This discrepancy may depend on both the temporal and quality of stress employed; moreover differences in the animal strains and species might also play a role.

Pregnancy chronic stress exposure does not affect adrenal glands plasticity

Several changes induced by the specific pregnancy chronic stress paradigm performed in this study on lactation-associated HPA axis plasticity and behaviour have already been characterized. Specifically, this chronic stress model was shown to prevent basal hyper-CORT on LD 8; although stress levels after 60 sec forced swim exposure were not affected (Hillerer et al., 2011); similar to the findings reported above following the HF diet. Therefore, I started my investigation assessing chronic stress effects on adrenal lipid droplets and LDLR expression; parameters that were shown in the previous chapter to undergo pronounced plasticity at mid-lactation and that were disrupted by HF diet. Virgins non-stressed animals were included in the study to confirm the lactation-associated changes while stress effects were only investigated in lactating rats. Stress exposure did not affect either of these parameters. Indeed, lipid droplets were decreased and LDLR expression increased in stressed mothers as in unstressed dams compared to virgins; no changes in absolute adrenal weight were observed. Thus, whilst stress exposure induced

a behavioural phenotype and HPA axis-associated changes that show similarity with the effects of feeding a HF diet, adrenal glands outcomes diverge.

HF diet and stress: Implications for basal hyperCORT and stress-hyporesponse in lactation

Interestingly, taken that mothers stressed with this specific stress paradigm were shown to display hyporesponse in terms of CORT secretion after 60 s forced swim without changes in ACTH (Hillerer et al., 2011), depletion of the adrenal cholesterol stores observed in stressed mothers further confirms the hypothesis that adrenal lipids depletion might underlie stress hypo-responsiveness at mid-lactation. On the other hand, HF diet-induced prevention of maternal basal hyperCORT correlated with altered adrenal plasticity; this was not the case in stressed mothers although stress effects on adrenal SRB1 expression need still to be addressed. However, the opposite impacts of diet induced obesity and chronic stress models on maternal metabolism might explain this discrepancy. Specifically, diet-induced obesity, as confirmed in this chapter, often associates with increased body weight and dyslipidemia characterized by increased tryglicerides and free fatty acids, decreased HDL cholesterol with HDL dysfunction and normal or slightly increase in LDL cholesterol with increased small dense LDL (Klop et al., 2013). Contrarily, chronic stress exposure decreases body weight gain and induces changes in plasma lipid profile that differ from the ones observed in obesity. For example, female mice exposure to combined acoustic and restraint stress on four successive days resulted in reduced body weight gain and plasma triglyceride levels whereas total cholesterol concentrations were increased. Plasma HDL concentration was higher while neither VLDL concentration nor LDL cholesterol levels changed (Depke et al., 2008). In male mice, chronic psychosocial stress exposure was reported to increase plasma LDL

cholesterol and to reduce liver triglyceride levels and body weight gain (Slattery et al., 2012, Czech et al., 2013, Fuchsl et al., 2013). Particularly regarding the peripartum period, the effects of pregnancy chronic stress exposure on maternal plasma lipids profile are still unknown. However, the reduction in body weight of stress mothers was shown to persist more than 13 days after stress termination thus suggesting that potential changes in plasma lipid profile might still be in place at that time or that they are reversed as are any potential adrenal changes occurring before LD 8 when the stress effects were assessed. Therefore, deficit in adrenal cholesterol supply might underlie chronic stress prevention of maternal basal hyperCORT.

In this study I revealed important effects of high fat diet feeding on the maternal adrenal and HPA axis related physiology. Indeed, diet-induced obesity was shown in the present chapter to prevented most of the lactation-associated changes in adrenal cholesterol delivery and the maternal basal hyperCORT, effects that were accompanied by maladaptative changes in maternal and anxiety-related behaviour of the dam. Taken together, these findings represent the first characterization of the HPA axis function in the obese rat dam and mechanisms that might underlie the obesity-associated HPA axis dysbalance have been identified. Although both pregnancy chronic stress and HF diet feeding models prevent maternal basal hyperCORT, the underlying mechanisms seem to diverge, at least concerning adrenal cholesterol delivery.

Chapter 4: Lactation-associated changes in adrenal cholesterol delivery in the mouse

4.1. Introduction

Having uncovered substantial peripartum-associated changes in the rat adrenal gland, I next wanted to question the translational significance of these findings. Thus, I hypothesised that assessing adrenal morphology in another species would represent a step towards the understanding of the physiological relevance of the findings described in the present thesis. Since similar peripartum changes in HPA axis-associated hormones have also been documented in the mouse, particularly regarding basal hyper-CORT (Douglas et al., 2003), I wanted to determine whether the adrenal glands adapt peripartum in this species.

Despite fewer studies focussing on HPA-axis related peripartum physiology in the mouse, evidence of pronounced plasticity has been documented. Douglas and colleagues provided hints about the peripartum hormonal profile in mice in a study assessing plasma ACTH and CORT at late-pregnancy and during lactation in outbred MF1 (BK white) mice. They revealed that plasma ACTH and CORT were not affected in L mice (from LD 2 to LD 5) compared to V; neither under basal conditions nor after exposure to stress (novel environment), while plasma ACTH, but not CORT, was decreased in L mice after forced swim. However, pregnant mice (PD 17-18) were shown to exhibit basal hyper-CORT. Although basal ACTH levels were not different among the groups, the ACTH increase after stress exposure (both novel environment and forced swim exposure) was attenuated in late-pregnant mice when compared with V animals (Douglas et al., 2003). Thus, the changes in HPA axis *in vivo* function at mid-lactation reported in this study are not

pronounced; finding that diverges from the profound lactation-associated plasticity documented in the rat discussed in the previous Chapter 2 and by others (Brunton et al., 2008, Hillerer et al., 2011, Hillerer et al., 2012).

Another study revealed that the stress-induced hormonal response in lactation was highly strain- and stressor-dependent. Specifically, inbred A/J mice showed diminished CORT response to electric shock compared to V on LD 5, but not on LD 12. In contrast, plasma CORT in C57BL/6J mothers was lower after shock on LD 12 but not on LD 5 (Hennessy et al., 1980a). However, no changes in basal CORT were revealed for none of the mice strain and time point examined. Interestingly, these two mice strains showed differences in maternal behaviour after infantile handling, while maternal care prior to experimental manipulation did not change across the groups (Hennessy et al., 1980b). Thus, stress-related changes in CORT might correlate with the previously mentioned behavioural outcomes.

Taken together, these findings suggest that maternal ACTH and CORT levels at basal conditions are less affected during lactation in the mouse contrary to what is observed in the rat. Indeed, whereas in the rat the maternal CORT levels are increased throughout the first two weeks after birth, in the mouse the hormonal HPA axis-associated changes seem to be more pronounced at late pregnancy and across the transition phase between pregnancy and lactation. In keeping, expression of the BMAL1 gene, an important component of the circadian clock, has been shown to be up-regulated in the mouse mammary gland, liver and adipose tissue the day after birth (Casey et al., 2009). Taken that BMAL1 gene expression has been shown to vary across several tissues after birth, it is likely that this might happen also in the adrenal glands where changes in BMAL1 have already been shown to correlate with adaptations in cholesterol delivery pathways (Leliavski et al., 2013).

Therefore, the present study was designed to investigate whether the adrenal glands show plasticity in the mouse during the first week after parturition. The main focus of the present study was the adrenal cholesterol delivery pathways; since I found that this important component of the steroidogenic machinery dramatically adapt peripartum in the rat. Confirmation of adrenal plasticity across species would strengthen the importance of the revealed adrenal changes for the overall HPA axis peripartum-associated function.

4.2. Materials and methods

4.2.1. Animals

Female C57BL/6 mice (four weeks old; Charles River, Sulzfeld, Germany) were left undisturbed for one week after arrival under standard housing (three to four mice per standard mice polycarbonate cage) and environmental (12h light-12h dark, lights on at 06:00, 22 ± 1° C, 60 ± 5% humidity) conditions. All experimental procedures were approved by the Committee on Animal Health and Care of the local government of the Oberpfalz and complied with international guidelines on ethical use of animals.

4.2.2. Experimental design

To assess adrenal lactation-associated changes in cholesterol delivery pathways, two cohorts of V and L mice at LD 1/2 (hereafter LD 1) and LD 8, respectively, were included in these studies (Fig. 38). All animals were killed by decapitation (08:00-10:00) after brief isoflurane anesthesia on the designated day; for each killing day, L and V control mice were killed together to minimize the environmental influence on the parameter of interest across the groups. Adrenal glands and trunk blood were collected and handled as following described.

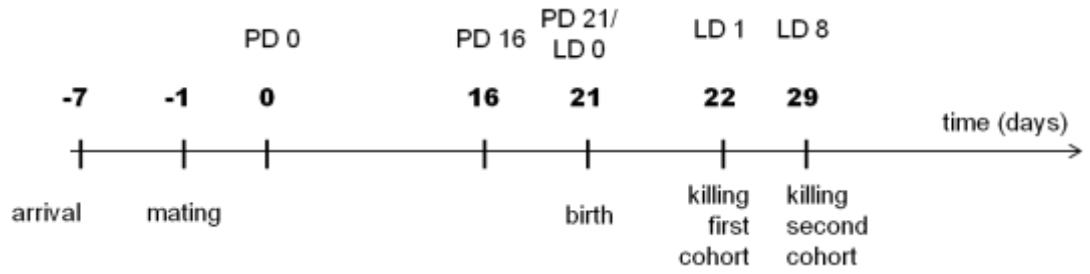


Figure 38. Lactation-associated plasticity in adrenal cholesterol delivery in the mouse: Experimental design

One week after arrival, animals were mated for one week and left undisturbed until single housing 17 days after mating started. Animals from the first experimental cohort were killed on LD 8 while animals from the second cohort were killed on LD 1 and plasma and adrenal glands collected.

N group numbers for the investigated parameters are reported in the following table.

Table 5. Lactation-associated changes in hormonal and adrenal parameters in the mouse: n numbers

	V	LD 1
Basal plasma ACTH	8	6
Basal plasma CORT	8	5[a]
Adrenal lipid droplets	8 [6 vs. LD 8]	6 [8 exp. LD 8]
Adrenal LDLR protein expression	6	6
Adrenal SRB1 protein expression	6	6
Adrenal HMGCR protein expression	4[b]	6
Adrenal HSL protein expression	6	6

a) One value excluded due to an ELISA problem
b) Two virgin samples were not analysed because the amount of isolated protein was insufficient for more than three blots

4.2.3. Mating and verification of pregnancy

For mating, female mice were housed with an experienced male (two females per male) for one week. To rule out any possible effect of mating on subsequent readouts, non-pregnant mice were assumed to be nulliparous (equates to V in the following text). On PD 17 mice were single housed to allow undisturbed birth and nursing of the offspring. On the day of parturition, defined as LD 0, due to high variability of the number of pups *per* litter and of the day of parturition (mothers gave birth over one week), pups were

distributed between mothers that delivered on the same day; each litter was composed by three to four pups.

4.2.4. Blood and organ preparation

Trunk blood was centrifuged and plasma aliquots were stored as previously described until ACTH and CORT were assessed *via* ELISA measurement as described in Section 2.2.4. Each adrenal, after pruning from fat was weighed. The left adrenal gland was embedded in protective freezing medium and a series of five 8 µm cryo-sections from the mid-part of the adrenal glands was cut and then thaw-mounted onto pre-coated slides (8 µm slices containing both adrenal cortex and medulla 6 sections per slide). The right adrenal gland was snap-frozen in liquid nitrogen and stored at -80° C until proteins isolation for western blot analysis, which was performed as described in Chapter 2 (see Section 2.2 for all methodological and material-related details).

4.2.5. Oil red lipid staining

The 8 cryo-sections were stained with Oil-red-O in order to assess lipids vesicles and light microscopy images of the stained adrenals were collected and analysed. The ratio between the stained area (mm^2) and the adrenal cortex area (mm^2) were measured and the stained area was expressed as percentage of the adrenal cortex (relative lipids content per cortex area) as described in Section 2.2.5.

4.2.6. Western blot analysis

Adrenal proteins were isolated *via* lysis buffer supplemented with a protease inhibitor cocktail, protein concentration was determined (see Section 2.2.8 for details) and 25µg from each protein extract were separated *via* electrophoresis using 10% sodium dodecyl

sulphate-polyacrylamide gel and transferred onto a nitrocellulose membrane as described in Section 2.2.8 (Bio-Rad, USA). TBST at pH 7.6, supplemented with 5% milk powder, was used to block the membranes and to dilute the primary antibodies. Blots were incubated over night at 4°C with the specific primary antibody against either LDLR, SRB1 or HMGCR. For secondary antibody incubation, membranes were incubated at room temperature for one hour with peroxidase-conjugated anti-rabbit IgG in TBST. Blots were washed with TBST after each antibody incubation session. β-tubulin was used as total protein loading control. Secondary antibody incubation was performed as described above. Bands were visualised using ECL western blot detection reagents and images were acquired and analyzed (for full methodological details see Section 2.2.8).

4.2.7. Statistics

Results were analysed using a two-way ANOVA statistical analysis and two-sided Student's t-tests and $P<0.05$ was considered significant. Statistical analyses were performed using IBM SPSS (version 21; Chicago, USA).

4.3. Results

4.3.1. Basal ACTH and CORT levels in virgin and lactating mice

Student's t test statistical analysis revealed that the reproductive status did not affect plasma ACTH (Fig. 39 A) whereas plasma CORT was shown to be significantly higher in the LD1 group compared to V (t_{11}=3.11; P < 0.05;Fig. 39 B).

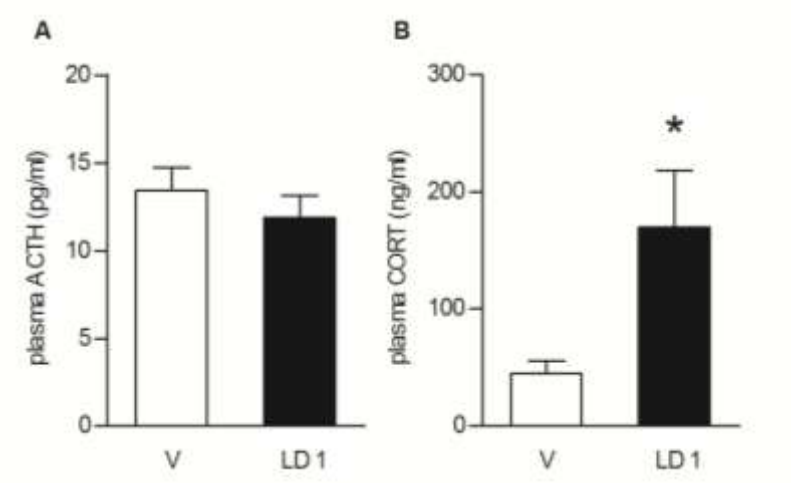


Figure 39. Basal plasma ACTH was not affected on lactation day (LD) 1 whereas CORT was increased compared with virgin (V) mice

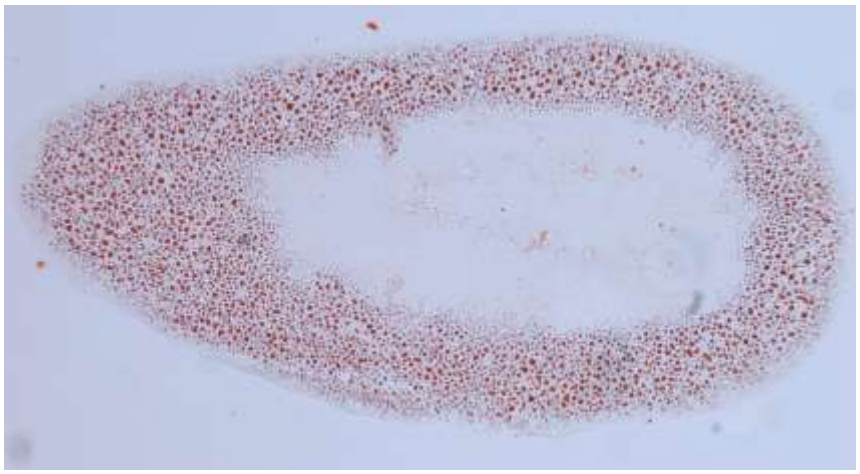
Basal plasma ACTH (A) and CORT (B) levels in V and L mice are represented. Despite no changes in ACTH, basal plasma CORT was increased on LD 1. Data represent mean + SEM (n=5-8 per group; see tab. 5 for the exact n numbers). * P<0.05 vs. V.

4.3.2. Adrenal weight and lipid droplets

Two-way ANOVA analysis revealed that neither the status nor the adrenal side affected the absolute adrenal weight (V left: 0.0029±0.0002; V right: 0.0025±0.0002; L left: 0.0025±0.0003; L right: 0.0023±0.0002 g).

Student's t test statistical analysis reported that the adrenal lipid droplets content (Fig. 40) did not differ between V and L animals neither on LD 1 (V: 100 ± 6.36; LD 1: 112.94 ± 20.75, % area stained of cortex area relative to V) nor on LD 8 (V: 100 ± 7.81; LD 8: 130.50 ± 18.85, % area stained of cortex area relative to V).

Figure 40. Representative picture of a stained adrenal gland slice from a virgin mouse



Six adrenal slices per animals were stained and lipid droplets content was assessed using a semi-quantitative measurement of the ratio between the stained area (mm^2) and the adrenal cortex area (mm^2).

4.3.3. Adrenal protein expression

Statistical analysis of adrenal protein expression showed that the reproductive status of the animals influenced LDLR, SRB1 and HMGCR expression. Specifically, both LDLR (t_{10}=2.42; P<0.05; Fig. 41 A) and SRB1 (t_{10}=3.97; P<0.01 Fig. 41 B) adrenal protein expression was increased in LD 1 mice compared with V. Also HMGCR was affected with the enzyme being highly expressed on LD 1 compared with V (t_8=2.33; P<0.05; Fig. 41 C) whereas HSL expression did not vary (Fig. 41 D).

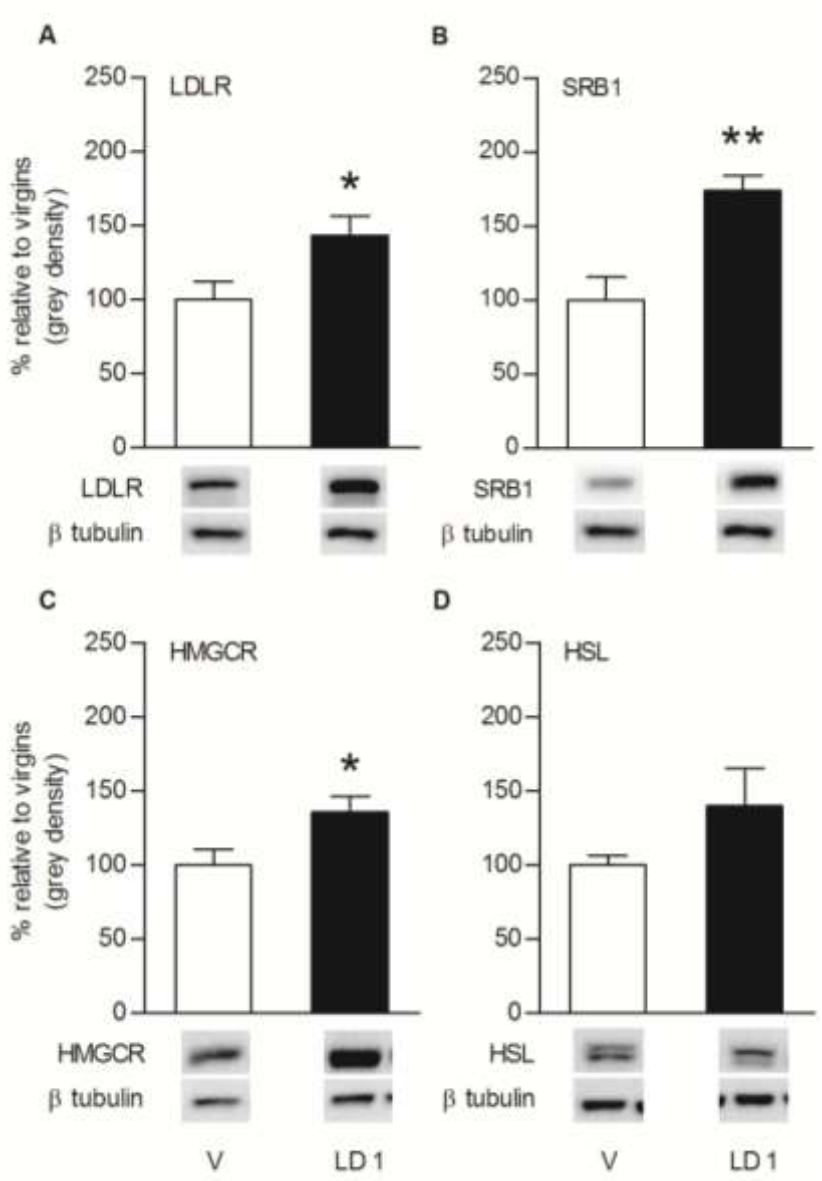


Figure 41. Adrenal LDLR, SRB1 and HMGCR expression is increased in lactating mice on lactation day (LD) 1 compared with virgins (V) whereas HSL is not changed

Western blot analysis was performed to assess whether adrenal protein expression is affected by the reproductive status in the mouse. A lactation-associated increase in LDLR (A), SRB1 (B), HMGCR (C) protein expression was observed. HSL adrenal (D) levels did not vary across the two groups. Data represent mean + SEM (n=5-6 per group; see Tab. 5 for the exact n numbers) from protein analysis which were normalized to the loading control, β-tubulin. * P<0.05, * * P<0.01 vs. V. Representative blots are shown below the corresponding graphs (upper bands) together with the loading control β-tubulin (lower bands).

4.4. Discussion

The aim of the present study was to examine ACTH and CORT levels after birth and whether adrenal cholesterol delivery pathways show plasticity in the lactating mouse. Overall, plasma CORT levels were increased on LD 1; despite plasma ACTH not differing between the groups. Furthermore, plasma lipoprotein receptors and HMGCR adrenal protein expression was increased on LD 1 compared with V whereas no differences in adrenal cortical lipid droplets content were detected at either time point. Taken together, these findings suggest that adrenal plasticity might participate in the mediation of the HPA axis hormonal-associated changes observed on LD 1 in the mouse. These finding confirm that adrenal glands adapt in the peripartum period across species and strengthens the hypothesis that adrenal adaptations may, at least in part, underlie the peripartum-associated basal hyper-CORT and stress hypo-responsiveness.

Analysis of the HPA axis-associated hormonal profile revealed that basal CORT levels were increased on LD 1 despite no changes in plasma ACTH. This finding suggests, therefore, that other factors beyond ACTH might be in place to stimulate the high adrenal steroidogenesis observed at this time. As discussed in Section 2.1, the elevation in the peripheral levels of plasma OXT that characterizes the physiology of the lactating mother across species might play a role in the CORT elevation; indeed, indirect evidence of OXT stimulating effect on adrenal steroidogenesis has been reported (Stachowiak et al., 1995). On the other hand, ACTH involvement cannot be completely excluded since ACTH-R adrenal expression and/or function might be changed, parameters that were not assessed in the present study. Such an increase in ACTH-R expression or function could consequently potentiate ACTH signalling in the adrenals.

While the reproductive status did not affect cortical lipid droplets, western blot analyses showed that adrenal LDLR, SRB1 and HMGCR expression levels were increased on LD 1

compared with V. These changes in adrenal proteins suggest that cholesterol uptake from plasma lipoproteins and *de novo* synthesis might represent the cholesterol sources preferentially recruited to sustain the high basal CORT production observed at this time. Indeed, primary roles of LDLR and SRB1 in both basal and ACTH-induced CORT production have been extensively speculated. For example, KO of the gene encoding for HSL, enzyme responsible for the hydrolysis of cholesterol esters provided *via* selective cellular uptake through SRB1 or from the adrenal lipid stores (see Section 1.2.2) was reported to reduce by 50% the basal CORT secretion from isolated adrenal cells *in vitro* whereas the CORT production induced by dibutyryl cAMP was reduced about 80% in the HSL KO mice (Kraemer et al., 2004). Therefore, it is likely that also changes upstream the HSL would impact on adrenal steroidogenesis. Interestingly, both LDLR and SRB1 expression was increased in HSL KO mice at basal conditions and measurement of the CORT levels *in vivo* revealed that female HSL KO mice had lower CORT compared to the wild-type mice whereas in male mice no effects of the HSL gene KO were visible (Kraemer et al., 2004). Therefore, at least in the male mice, compensatory mechanisms prevented the HSL KO-induced changes in the CORT *in vivo* levels. The author of the previously mentioned study theorizes that the HSL KO impact on the CORT levels *in vivo* are related to ACTH-induced CORT secretion, although the hormone measurements were performed in blood that was collected one hour after an ACTH injection. Therefore, given that ACTH-induced CORT is normally back to basal levels one hour after injection as sown in Section 3.3.4.2 of the present thesis, it is likely that the CORT changes observed in HSL KO mice refer to basal CORT concentrations. Therefore, taken together these results highly suggest that the LDLR-mediated uptake and the *de novo* cholesterol synthesis might buffer the HSL KO effects *in vivo* in male mice. The gender differences in the Kraemer study might involve changes in the *de novo* cholesterol synthesis regulation, issue that was unfortunately not investigated by the author. Increased LDLR and SRB1 protein levels might represent an adrenal attempt to cope with challenging situations like

the one imposed by the KO of the HSL gene, changes that mirror the receptors profile observed on LD 1 in this study. Therefore, it is likely that the sustained basal hyper-CORT production that the maternal adrenal glands cope with throughout the peripartum period represent a challenge that has already been shown to induce profound plasticity in the adrenal physiology and structure in the rat (see Chapter 2).

Despite the increase in adrenal SRB1 expression, HSL expression was unchanged on LD 1; although a strong trend towards this was observed. However, since HSL hydrolytic activity is controlled by phosphorylation and by oligomerisation, with HSL being more active when phosphorylated by PKA and when in dimers (Garton and Yeaman, 1990, Shen et al., 2000), it is possible that HSL activity might be increased without changes in the total protein expression. Future studies should be performed to further elucidate HSL and ACAT participation to sustain basal hyperCORT.

Adrenal lipid droplets were not altered on LD 1 or LD 8; therefore, mice adrenal glands seem to predominantly rely on plasma cholesterol up-take and *de novo* synthesis to sustain basal hyper-CORT even when adrenal cholesterol stores are available. These findings, together with the demonstration that stress hypo-responsiveness is consistently shown in the rat across studies whereas the results from mice studies are more controversial, confirm that lipid droplets availability underlie an adequate CORT response to acute stress exposure. For example, Douglas and colleagues showed that the CORT response to acute stress exposure did not differ between V and L (from LD 2 to LD 5) mice (Douglas et al., 2003). Therefore, it is plausible that mothers, with intact adrenal cholesterol store availability, could mount the same CORT response as V mice did. It would be interesting to assess adrenal cholesterol availability at late pregnancy when CORT response to novel environment and forced swim exposure was reported to be blunted in the pregnant mouse (Douglas et al., 2003) or on LD 12 when BL6 mothers were

shown to display lower CORT levels after shock exposure compared to V (Hennessy et al., 1980b). Further, it would be necessary to determine whether stress response is affected on LD 1. It would also be interesting to study HPA axis plasticity in HSL and LDLR KO mice to clarify their importance for the overall HPA axis function *in vivo*.

As mentioned in the introduction of this Chapter, BMAL1 mRNA expression was shown to vary after birth in several tissues like that mammary gland, liver and adipose tissue and these changes are hypothesised to represent a mechanisms through which different maternal tissues coordinate their activity to face with the challenge of lactation. However, to my knowledge, BMAL1 expression has never been measured in the adrenal glands although the finding reported in the present Chapter highly suggest that its expression might vary also in the adrenals. Indeed, given that the KO of the gene encoding for BMAL1 has been reported to markedly impact on adrenal cholesterol delivery, it is likely that on LD 1, when these pathways have been shown in this Chapter to highly adapt, changes in BMAL1 regulation might participate. Therefore, given the important role of the adrenal glands as the peripheral circadian pacemaker, adrenal plasticity is hypothesised to be necessary for the overall HPA axis fine tuning after birth and throughout lactation. My evidence of adrenal plasticity in both rats and mice, albeit through different mechanisms, strengthens the possibility that peripartum adrenal plasticity might occur even in humans highlighting the importance of the findings from animal models to reveal the mechanisms of the peripartum-related HPA axis plasticity.

Chapter 5: General Discussion

The aim of the present thesis was to assess whether the adrenal glands undergo plasticity during pregnancy and lactation and whether such changes may relate to the maternal HPA axis-related hormonal profile under basal and stress conditions. Indeed, despites the crucial involvement of the adrenal glands in the regulation of plasma CORT levels, to the best of my knowledge, the study of HPA axis-associated peripartum plasticity was confined at the brain and pituitary level so far. I could demonstrate several indications of marked adrenal plasticity in the present thesis across the peripartum period but particularly during lactation, when the changes in basal HPA axis function are sustained. Specifically, whereas the changes in adrenal cholesterol delivery were limited to a substantial depletion of the adrenal cortical lipid droplets depletion, depletion that has been hypothesised to participate to the maternal stress hyporesponsiveness, at mid-lactation this reduction of the adrenal cholesterol stores was accompanied by increased expression of the plasma lipoproteins receptors SRB1 and LDLR and by a decrease in HMGCR protein levels. The changes I observed appear to be particularly robust given that most of the adaptations were observed in both rats and mice and across numerous cohorts. Moreover, the present investigation of estrous cycle-associated changes in cholesterol delivery revealed that reproductive experience rather than estrous cycle-associated transient hormonal changes markedly impact on adrenal glands plasticity. This plasticity is likely to directly affect adrenal function, taken that diet-induced obesity prevented most of the lactation-associated adrenal changes and was associated with HPA axis dysregulation *in vivo*. On the other hand, chronic stress during pregnancy, which has previously been shown to prevent maternal basal hyperCORT on LD 8, did not affect any of the adrenal parameter assessed. Therefore, whilst HF diet intake and chronic stress prevented maternal basal hyperCORT the underlying mechanisms seem to diverge, at least at the adrenal level.

Cholesterol balance is important for adrenal function peripartum

Maternal cholesterol plasma levels and hyperlipidemia are known to crucially influence embryonic development and fetoplacental growth. Indeed, altered cholesterol placental supply to the foetus is believed to concur to intrauterine growth retardation (IUGR) since placental expression of LDLR and SRB1 has been shown to be altered in women that are diagnosed with IUGR, changes in placental lipoproteins receptors expression that were accompanied by reduced circulating LDL (Wadsack et al., 2007). Therefore, besides the importance of maternal cholesterol balance for placental function and foetal development, my results suggest that optimal cholesterol availability is fundamental also for the peripartum adrenal glands function. Further, the possibility that changes in adrenal cholesterol supply might dysregulate the overall peripartum HPA axis-related that, in turn, increases the risk for the development of stress-related disorders should be kept in mind when the use of drug to treat dyslipidemia during pregnancy is considered. Indeed, although the use of HMGCR inhibitors (statins) to treat dyslipidemia is contraindicated during pregnancy, the circulating cholesterol levels or the pregnancy stage at which the risk of hypercholesterolemia is lower than the risk derived from statins is still unknown and, therefore, some patients do not interrupt the therapy when pregnant (Lecarpentier et al., 2012). However, it should be considered that besides the already hypothesised, and partially proven, statin-related interference with physiological placental development (Kenis et al., 2005) and their teratogenic effects (Godfrey et al., 2012), these drugs might alter the fine-tuned peripartum adrenal cholesterol dynamics that have been highlighted in the present thesis.

New insights into the physiological relevance of adrenal receptor-mediated cholesterol endocytosis

In the previous chapters I have shown that LDLR expression in the female rodent adrenal is highly plastic, particularly when basal steroidogenesis is increased (see Sections 2.3.1.2 and 2.3.3.5). Although the increase of SRB1 and LDLR in lactating rats would suggest that both receptors are important to sustain maternal basal hyperCORT, the study of the impacts of HF diet feeding on adrenal morphology and HPA axis activity at mid-lactation reported in Chapter 3 suggests a prominent role of LDLR-mediated uptake in this effect. Indeed, the lactation-associated increase in LDLR was blocked in HF diet mothers without changes in SRB1, normalization of the adrenal LDLR expression to V levels that were accompanied by basal hyperCORT prevention. Taken together, these findings shed light on the physiological meaning and implication of the endocytic cholesterol uptake in the rodent adrenal, cholesterol source whose contribution was largely unclear so far. Specifically, whereas the importance of the SRB1-related cholesterol supply in the rodent adrenal is well understood (Miller and Bose, 2011), the exact contribution of the uptake through the LDLR was still ambiguous. Indeed, as previously mentioned, the KO of the gene encoding for HSL has been shown to result in a strong reduction in both basal- and ACTH-induced steroidogenesis *in vitro* (Kraemer et al., 2004). In contrast to these strong effects, the KO of the LDLR gene in mice has been reported to neither affect the basal nor the ACTH-induced CORT production from adrenal cells isolated *in vitro* (Kraemer et al., 2007). Therefore, the data reported in the present thesis suggest that adrenal endocytic cholesterol uptake is markedly involved to sustain basal steroidogenesis, particularly when basal CORT secretion is high. Understanding of the LDLR-associated physiology is particularly relevant with respect to the human condition taken that, contrary to what happened in rodents, LDLR mediated endocytosis appears to play a crucial role to sustain steroidogenesis in the human adrenal. Indeed, addition of LDL, but not of HDL, to the

culture medium of untreated human adrenal cells (without ACTH) has been shown to increase basal CORT production. Addition of ACTH to the cell culture increased both LDLR and CLA-1 (human omolog of the rodent SRB1) mRNA in human adrenal cells with the increase of the mRNA for LDLR being faster; LDLR mRNA was, indeed, significantly increased already after two hours of ACTH treatment whereas the effects on CLA-1 mRNA took longer (Liu et al., 2000). Moreover, my results confirm that the expression of the LDLR is more sensitive than that of SRB1 gene to changes in cholesterol availability, which is similar to what has been shown for CLA1 in humans (Liu et al., 2000). Thus, taken the higher impact of the LDLR activity for adrenal steroidogenesis in humans, animal models characterized by an important participation of the LDLR might better mimic the human condition and, therefore, represent a suitable method to study adrenal cholesterol dynamics. In that light, more study of the effects of diet induced obesity during lactation in the rat may shed light on the physiological importance of the changes in adrenal cholesterol delivery.

The adrenal glands contribution to sustain basal hyperCORT differs across pregnancy and lactation

Besides highlighting the LDLR importance for peripartum adrenal cholesterol supply and the participation of lipid droplets depletion to the maternal stress hyporesponsiveness, the *in vitro* findings reported in Section 2.3.2.3 of the present thesis suggest that the mechanisms underlying maternal basal hyperCORT might diverge across pregnancy and lactation. Indeed, incubation of adrenal cells from LD 8 animals resulted in hyperCORT secretion compared to cells from V animals *in vitro*; a result that was entirely adrenal mediated and mirrors the *in vivo* condition. On the other hand, CORT secretion from adrenal cells from PD 21 animals was not increased, although *in vivo* CORT plasma

levels are already high at that time but adrenal plasticity is limited to adrenal lipid droplets depletion and the expression of both LDLR and SRB1 is unchanged compared to V animals. Thus, without any further external stimuli, adrenal glands from LD 8 animals can independently maintain high CORT levels while at PD 21 extra adrenal factors must be involved. As already discussed in Chapter 2, estrogens represent such candidates since they rise during pregnancy and influence adrenal steroidogenesis (Nowak et al., 1995, Di Croce et al., 1999, Lo et al., 2000, Figueiredo et al., 2007, Brunton and Russell, 2010); although direct evidence of estrogens modulation of the adrenal function peripartum is still missing.

The importance of studying adrenal plasticity across the estrous cycle

The estrous cycle-associated investigation described in Chapter 2 highlighted how transient hyperCORT states like the proestrous-associated CORT changes in cycling females do not require substantial plasticity of the adrenal cholesterol delivery pathways. Thus, estrous-cycle associated adrenal changes were limited to an increase in LDLR expression at estrous, an increase that might, therefore, account and satisfy the transient increase in cholesterol demand at that time. However, taken that HF diet-induced prevention of lactation-associated adrenal plasticity was shown to correlate with altered HPA axis function, it would be interesting to test whether interfering with adrenal cholesterol recruitment across the estrous cycle might impact on HPA axis function in cycling females. To address this issue, longer diet exposure or higher fat concentrations might help to exacerbate diet-induced effects on adrenal physiology in virgins. Indeed, all the HF diet-induced changes described in the present thesis were lactation-specific, finding that confirms the peripartum period as particularly susceptible to the environmental conditions. This analysis is relevant to approach pathologies like the premenstrual

dysphoric syndrome that is characterized by altered HPA axis activity. Indeed, women suffering from this syndrome show lower basal evening CORT concentrations and increased CORT response to ovine CRH when compared to healthy controls (Rabin et al., 1990). Intriguingly, this hormonal profile highly resembles the CORT pattern observed in HF diet fed mothers that were characterized by prevention of the basal hyperCORT and by an exaggerated response to ACTH injection. Studies performed with HSL or LDLR KO cycling females could address these issues.

High-fat diet feeding and pregnancy chronic stress HPA axis-associated dysbalance and behavioural outcomes

Although both HF diet feeding and pregnancy chronic stress prevented maternal basal hyperCORT on LD 8 and increased anxiety-like behaviour of the dam as shown in Sections 3.3.2, 3.3.4 and others (Hillerer et al., 2011), the impact of these two animal models on adrenal morphology diverge. Although chronic stress did not affect adrenal lipid droplets or LDLR expression, it is important to consider that these measurements have been performed more than 12 days after stress termination; increasing the probability that compensatory mechanisms might be in place at that time. Moreover, it is still unclear whether pregnancy chronic stress affects adrenal SRB1. Future studies might clarify whether chronic stress impact on the pregnancy-associated adrenal dynamics at different time points and on other parameters.

The effects of feeding a HF diet on maternal adrenal plasticity and basal hyperCORT reported in the present thesis have been shown to be associated with changes in maternal- and anxiety-related behaviour (see Section 3.3.2). On the other hand, the behavioural consequences of the pregnancy chronic stress model described in Chapter 3

have already been investigated by others (Hillerer et al., 2011). The behavioural effects of the diet-induced obesity model established with the present thesis confirm this paradigm as a reliable animal model to study the comorbidity between obesity and anxiety disorders whose underlying mechanisms are poorly understood, particularly concerning the peripartum period. However, given that obesity is also a known risk factor for the development of depression, it would have been interesting to test whether diet-induced manipulation impacts on the depressive-like behaviour of the dam. However, this hypothesis was not assessed in the present thesis taking several concerns about the reliability of the test available to assess depressive-like behaviour in obese animals. Indeed, the higher body weight of the obese mothers might influence *per se* the time that the rat spends immobile during the forced swim test, the most validated test to assess depressive-like behaviour in rodents (Cryan and Slattery, 2007). Also the use of the sucrose preference test to assess anhedonia, a core symptom of depression, is discouraged taken that the high maternal energy demand might influence *per se* sucrose intake. The dual-choice conditioned place preference procedure, a test that has been established to study the motivational processes underlying maternal behaviour might further clarify the effects of chronic stress or HF diet feeding on maternal behaviour. Indeed, healthy dams have been reported to display higher preference for pup cues versus cocaine-associated cues (Mattson and Morrell, 2005). This preference might be altered in obese/stressed mothers; however, this hypothesis has been investigated so far. However, it is important to mention that postpartum anxiety and depression are often comorbid and, therefore, the possibility that they share part of the underlying pathophysiology is probable.

Relevance of the findings of the present thesis

The peripartum-associated changes in adrenal cholesterol delivery pathways reported in the present thesis seem to be robust adaptations taken that these changes were observed in several animal cohorts and in both rats and mice. On the other hand, confirmation of the present findings from future studies performed in other laboratories or in different strains and species would further strengthen the physiological meaning of the adrenal peripartum-associated changes that have been revealed with the present thesis. Moreover, it is important to consider that the differences in the adrenal steroidogenic enzymatic pools of rodents and humans described in Section 1.2.1.1 are downstream of cholesterol recruitment and transport to the inner mitochondria membrane. Therefore, the peripartum-associated adrenal plasticity observed in rats and mice involve components of the steroidogenic machinery that do not differ between humans and rodents and this increases the possibility that the peripartum-adrenal findings described in the present thesis might be translational to humans.

5.1. Outlooks

The new insights into adrenal plasticity peripartum and into the effects of diet induced obesity and pregnancy chronic stress on adrenal adaptations and peripartum HPA axis activity described in the present thesis opened a series of research scenarios that might be investigated with future studies.

First of all, in the present study, the adrenal glands have been shown to undergo substantial plasticity peripartum and prevention of most of these changes were associated with HPA axis dysbalance and increased anxiety like behaviour in the dam. Therefore, it

would be interesting to assess whether peripartum-associated adrenal morphology and function differ between rats selectively bred for high and low anxiety-like behaviour, which also differ in their HPA axis reactivity.

As previously mentioned, the sympathetic innervation to the adrenal glands is known to prominently influence steroidogenesis and, therefore, it would be of high interest to assess if the sympathetic firing to the adrenals adapts peripartum. Indeed, contrary to the well described reduction of the central noradrenergic drive to the PVN in the rat peripartum (Douglas, 2005), pregnancy and lactation have been associated with peripheral sympathetic hyperactivity in women and rats (Greenwood et al., 2001, Jarvis et al., 2012), findings that suggest that the sympathetic firing to the adrenals might also be increased. However, the peripheral contribution of the sympathetic nervous system to the modulation of HPA axis activity peripartum is still unknown. This could be addressed through *in vivo* measurements of plasma CORT levels and adrenal parameters after splanchnicectomy in pregnant and lactating rats or *in vitro* through adrenal cells stimulation with adrenaline and noradrenaline, for example. The *in vitro* approach mentioned above might also clarify the so far just hypothesised and previously described sex steroids and PRL involvement to sustain basal hyperCORT during pregnancy and lactation, respectively.

Furthermore, as reported in Section 2.3.3, I did not observe any indication of adrenal plasticity on PD 4 whereas on PD 13 the adrenal lipid droplets were already markedly depleted, time points when the basal hyperCORT was already fully manifested. Thus, investigation of adrenal plasticity between PD 4 and 13 would give further hints to understand whether adrenal plasticity triggers basal hyperCORT or *vice versa*. Moreover, the analysis of potential adrenal changes at early-lactation could help to clarify whether birth-related mechanisms trigger adrenal plasticity or whether labour-independent mechanisms are involved. Furthermore, as already mentioned, it would be interesting to

investigate the potential peripartum adaptations of other components of the adrenal steroidogenic machinery as well as HF diet feeding and pregnancy chronic stress effects on such parameters; among them StAR and the CYP450 and HSD enzymes. Further investigation of the hypothetical changes in ACTH-R function, sensitivity and of the pathways downstream the ACTH-R is also needed.

HF diet feeding was shown to markedly impact on adrenal peripartum plasticity, HPA axis *in vivo* function and behaviour in the rat. Therefore, the above mentioned HF diet feeding effects has to be confirmed in the mice to assess whether the observed diet induced obesity maladaptations are species specific. Moreover, whilst the central mechanism underlying the peripartum changes in basal and stress induced HPA axis activity have been extensively studied in the rat, little has focused on the plasticity occurring in the pregnant and lactating mouse. Particularly the plasticity of the AVP system seems to diverge taken that circulating AVP levels has been reported to be increased in the lactating rat (Suzuki et al., 2000) but decreased in the pregnant and lactating mouse (Douglas et al., 2003). Therefore, study of the central AVP system peripartum plasticity in the mouse should be addressed taken that this system has already been shown to highly adapt in the rat (Toufexis et al., 1999, Walker et al., 2001).

Moreover, as previously mentioned, changes in BMAL1 expression were shown to impact on adrenal cholesterol availability and HPA axis function (Leliavski et al., 2013) and BMAL1 mRNA levels are known to increase after birth in several tissues. Therefore, it would be interesting to assess whether BMAL1 expression is altered in the adrenals as well and whether peripartum-associated changes in the adrenal circadian clock influence basal and stress-induced steroidogenesis in rodents.

5.2. Summary - concluding remarks

The present thesis aimed to investigate whether the adrenal glands adapt peripartum to sustain basal hyperCORT, important maternal HPA axis-related adaptation, and to assess whether prevention of such changes might participate to the aetiology of postpartum pathologies that are characterized by e in HPA axis function. Several indications of adrenal plasticity have been highlighted, particularly at mid-lactation. Indeed, I have shown that, although adrenal lipid droplets were markedly depleted starting already at mid-pregnancy, all three adrenal cholesterol delivery pathways profoundly adapted at mid-lactation. Specifically, adrenal cortical lipid droplets depletion was accompanied by increased expression of both, LDLR and SRB1, whereas HMGCR protein levels were decreased. Most of the above described changes occurred in several animal cohorts and across species and, therefore, might represent robust peripartum adaptations; the observation that even chronic pregnancy stress exposure did not affect the adrenal changes in the parameters assessed strengthened this hypothesis. Intriguingly, adrenal plasticity was substantially impaired by HF diet intake, manipulation that also prevented basal hyperCORT and linked to an exaggerated response to ACTH intravenous injection. The changes in adrenal morphology and HPA axis function were accompanied by behavioural abnormalities with obese mothers showing decreased maternal care the day after parturition and increased anxiety-related behaviour. Importantly, similar behavioural and HPA axis-associated aberrations like maternal basal hyperCORT prevention and impaired mother-infant interaction are known features of human postpartum pathologies and, therefore, the animal models described in the present thesis might represent valid tools to study their pathophysiological mechanisms. Taken together, the findings reported in the present thesis shed light on adrenal mechanisms that are highly suggested to relate to the “peculiar” HPA axis peripartum function at both, physiological and pathological conditions.

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Abbreviations

ABN arched back nursing

ACAT acyl Coa cholesterol acyltransferase

ACTH adrenocorticotropic hormone

ACTH-R adrenocorticotropic hormone receptor

ANOVA analysis of the variance

AUC area under the curve

AVP vasopressin

BMI body mass index

cAMP cyclic adenosine monophosphate

CE cholesterol esters

CYP cytochrome P450

CYP11A1 or P450scc side-chain cleavage enzyme

CYP11B2 aldosterone synthase

CORT corticosterone

CRF corticotrophin-releasing factor

D diestrous

DMEM Dulbecco’s Modified Eagle medium

E estrous

ELISA enzyme-linked immunosorbent assay

ER endoplasmic reticulum

FS forced swim

FSH follicle-stimulating hormone

GnRH gonadotropin-releasing hormone

GR glucocorticoids receptor

HDL high density lipoproteins

HF high-fat

HMGCR 3-hydroxy-3-methylglutaryl-CoA reductase

HSL hormone sensitive lipase

HSD hydroxysteroid dehydrogenase

HPA axis Hypothalamus-Pituitary-Adrenal axis

inj injection

IUGR intrauterine growth retardation

KO knockout

L lactating

LD lactation day

LDB light-dark box

LDL low density lipoprotein

LDLR low density lipoprotein receptor

LD lipid droplets

LH luteinizing- hormone

M metestrous

mwu Mann Whitney U test

MR mineralocorticoid receptor

NF normal fed

NS non-stressed

OC overcrowding stress

OXT oxytocin

P proestrous

PD pregnancy day

PK protein kinase

PRL prolactin

PRL-R prolactin receptor

PPD postpartum depression

PVN paraventricular nucleus

PW 4 weeks post weaning

RAR receptor autoradiography

RS restraint stress

S stressed

s seconds

SEM standard error of the mean

SRB1 scavenger receptor class B type 1

StAR steroidogenic acute regulatory protein

TH tyrosine hydroxylase

TBST Tris-buffered saline/0.1% Tween-20

V virgins

VLDL very low density lipoproteins

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Regensburg, Februar 2014

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