



FACULTY OF HEALTH SCIENCES  
AARHUS UNIVERSITY

# Stress during fetal life and acoustic startle response changes in adult rat offspring

PhD dissertation

Sanna Lemming Kjær

Faculty of Health Sciences  
Aarhus University  
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Aarhus University  
Centre for Psychiatric Research

”And surely we are all out of the computation of our age, and every man is some months elder than he bethinks him; for we live, move, have a being, and are subject to the actions of the elements, and the malices of diseases, in that other World, the truest microcosm, the Womb of our Mother”

(Sir Thomas Browne, *Religio Medici*, 1642)

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**Dansk titel:** Stress under fostertilstanden og akustisk startle forandringer hos voksne rotteafkom

**Author:** Sanna Lemming Kjær, M.sc.

**Academic advisors:** Raben Rosenberg and Gregers Wegener, Centre for Psychiatric Research, Aarhus University. Karin Sørig Hougaard, National Research Centre for the Working Environment.

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**Review committee:**

Professor, dr.med. Per Hove Thomsen (Chairman), Psychiatric Hospital for Children and Adolescents, Risskov, Aarhus, Denmark

Associate professor David Paul Drucker Woldbye, Laboratory of Neuropsychiatry, Department of Neuroscience and Pharmacology, University of Copenhagen and University Hospital Rigshospitalet, Copenhagen, Denmark

Assistant Professor Tracy Doucette, Department of biology, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada

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## 1. Preface

The present thesis is based on experimental work performed at the National Research Centre for the Working Environment, the Centre for Psychiatric Research and University of Prince Edward Island during my employment as a PhD student at Aarhus University, June 2007- May 2010. This work would not have been possible without the generous help and assistance from a lot of people, fellow researchers, colleagues, friends and family, whom I would like to thank here. In particular I would like to thank:

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## 2. List of papers

The PhD thesis is based on the following original papers, which can be found in the appendix:

- Paper I)** **Kjær SL**, Wegener G, Rosenberg R, Lund SP, Hougaard KS (2010) Prenatal and Adult stress interplay - Behavioral implications. *Brain Res* 1320:106-13
- Paper II)** **Kjær SL**, Hougaard KS, Tasker RA, MacDonald D, Rosenberg R, Elfving B, Wegener G (2010) Influence of diurnal phase on startle response in adult rats exposed to dexamethasone in utero. *Submitted to Pharmacol Biochem & Behav*
- Paper III)** **Kjær SL**, Wegener G, Rosenberg R, Hougaard KS (2010) Reduced mobility but unaffected startle response in female rats exposed to prenatal dexamethasone – different sides to a phenotype. *Dev. Neurosci* x:x-x
- Paper IV)** Hougaard KS, Mandrup KR, **Kjaer SL**, Bøgh IB, Rosenberg R, Wegener G (2010) Effects of prenatal Chronic Mild Stress on the acoustic startle reaction in male rats *In manuscript*

### 3. Abbreviations

ASR	Acoustic startle response
ATCH	Adenocorticotrophic hormone
AVP	Adeno vasopressin
BNST	Bed nucleus of the striated terminalis
CMS	Chronic mild stress
CON	Control
CORT	Short for either cortisol (humans) or corticosterone (rodents)
CRH	Corticotrophin releasing hormone
CRHBP	Corticotrophin releasing hormone binding protein
CRHR1	Corticotrophin releasing hormone receptor 1
CRHR2	Corticotrophin releasing hormone receptor 2
DEX	Dexamethasone
5-HT	5-hydroxytryptamine (Serotonin)
GR	Glucocorticoid receptor
HPA	hypothalamic pituitary adrenal
MR	Mineral corticoid receptor
mRNA	messenger ribonucleic acid
PNM	Prenatally manipulated – used in this thesis to indicate exposure to either prenatal stress or prenatal dexamethasone
PNS	Prenatal stress

PPI	Prepulse inhibition
PPT	pedunculo pontine tegmental nucleus
PVN	paraventricular nucleus
RT-qPCR	Real-time quantitative polymerase chain reaction

## 4. Background

During the last decade there has been a rising awareness and acknowledgement of the problems of stress in our working environment. Increasing demands on performance, capability of handling constant changes, and achieving social success put people under a huge mental as well as physical strain and the rising number of stress related illnesses bear witness to this (Ljung and Friberg 2004). In Denmark alone, every tenth individual engaged in active employment reports a stressful daily life (Nielsen et al. 2004) and each year psychological work environment is the cause of 30.000 hospitalisations (Statens Institut for Folkesundhed 2005)

Inter-individual differences in coping with stress, at least partly, depend on the secretion and action of stress hormones, which are largely shaped by gene–environment interactions throughout life (Oitzl et al. 2009). Ineffectual coping, due to a genetic vulnerability or other predisposition which interact with the environment and stressful life events, may trigger mental disease.

Prenatal stress (PNS) has been associated with decreased adaptation to new situations and increased emotionality in humans (Meijer, 1985; Van den Bergh et al., 2005; Weinstock, 2001) and is a possible candidate for a predisposing condition for mental health effects later in life. In animals, PNS has been shown to increase the hypothalamic pituitary-adrenal (HPA) axis response to stress, often manifested as enhanced or prolonged corticosterone response to acute stressors (Henry et al., 1994; Koenig et al., 2005), and to increase emotionality in line with the human findings (Burton et al., 2006; Cabrera et al., 1999; Darnaudery and Maccari, 2008; Lehmann et al., 2000; Maccari et al., 2003; Mastorci et al., 2008). Prior to the start of this PhD, an animal model combining pre- and postnatal manipulations had been established at the National Research Centre of the Working Environment. Two studies, of which I took part in and co-authored one (Hougaard et al. 2005b) observed an association between prenatal chronic mild stress (CMS) or exposure to dexamethasone (DEX), postnatal blood sampling under restraint and increased basal startle in adult rats (Hougaard et al. 2005a; Hougaard et al. 2005b). The work in the current thesis is based on these two studies and aims to investigate the individual components of the prenatal and postnatal manipulations, and the association between them, which had led to the phenotype of

changed basal startle reactivity observed. Because of its potential translational value across species, modulation of startle reactivity may be very useful in examining altered emotional reactivity following prenatal insults (Bijlsma et al. 2010).

#### ***4.1 Stress***

Stress in humans may be defined as a threat, real or implied, to the psychological or physiological integrity of an individual (McEwen 2000). Stress is subjective, as individuals differ both in perception and coping ability, depending on prior experience, physical condition and personal behaviours (McEwen 2000; Thornton and Andersen 2006). Adding to the complexity, the stress response is not static, it changes over time as a function of the life history of the individual (Levine 2001; Ganzel et al. 2010). Not only humans but all animals, from wildlife to lab rats, can experience stress if their environment changes too much, their routines are interrupted constantly by intruders or the predator pressure increases. The terms allostasis and allostatic load provide a conceptual frame work to explain the complexity of the stress response. Allostasis means maintaining stability through change and represents the adaptational process of the body to physical, psychosocial and environmental challenges or stress; Allostatic load represents the physiological cost of the system changes caused by the adaptational response (allostatic accommodation), which ranges from being negligible to resulting in pathology and chronic illness (McEwen 2000; Logan and Barksdale 2008; Ganzel et al. 2010). For instance, hormones associated with stress can have protective effects in the short run but damaging effects if the exposure is of longer duration (McEwen 1998; McEwen 2000).

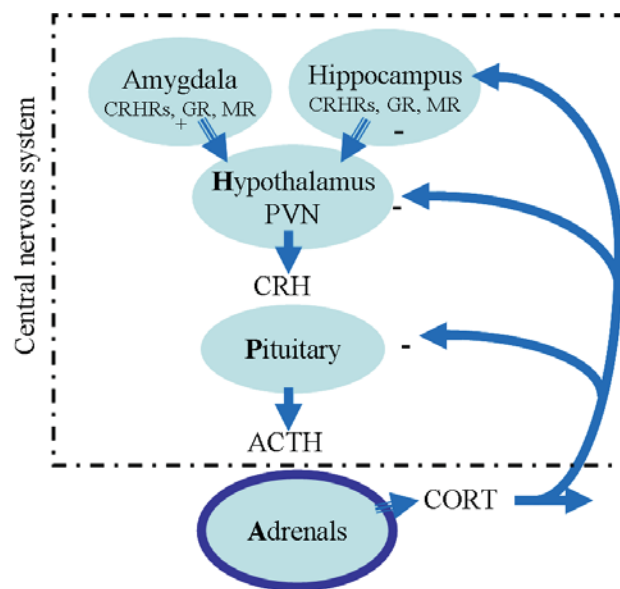
#### ***4.2 Stress response***

Exposure to hostile conditions results in a series of coordinated responses aimed at enhancing the probability of survival (Darnaudery and Maccari 2008). Located primarily in the central nervous system, two chief participants of the stress response are the sympathetic nervous system and the HPA axis system. The parallel engagement of components of the sympathetic nervous system with the HPA axis serves to coordinate endocrine and cognitive limbs of the stress response (Valentino and Van 2008). Activation of the sympathetic nervous system leads to elevated release of the catecholamines epinephrine

and norepinephrine as well as other transmitters (Dunser and Hasibeder 2009). The main effect of these is to prepare the body for a “fight or flight” response, which is done by increasing the heart rate and blood pressure in addition to providing fuel for the body by stimulating breakdown of fats and glycogen (Dunser and Hasibeder 2009)<sup>1</sup>.

#### 4.2.1 HPA axis

The HPA axis system involves release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) in the hypothalamus, leading to adenocorticotrophic hormone (ACTH) release from the anterior pituitary which in turn stimulates synthesis and release of glucocorticoids, cortisol (humans) or corticosterone (rodents) (CORT) from the adrenal glands (Meaney et al. 1996) (Fig. 1). CORT helps maintain basal activity of the HPA system and controls the sensitivity of the system’s response to stress, but also exerts a negative feedback on the HPA axis by way of the glucocorticoid (GR) and mineralcorticoid (MR) receptors in the hippocampus (De Kloet et al., 1998;Derijk and De Kloet, 2008;Oitzl et al., 2009).



**Figure 1:** A simplified representation of the hypothalamic-pituitary adrenal (HPA) axis system. CRH= corticotrophin releasing hormone; CRHRs; corticotrophin releasing hormone receptors GR= glucocorticoid receptor; MR= mineralcorticoid receptor; PVN= paraventricular nucleus.

<sup>1</sup> The sympathetic nervous system is not included in the remainder of this thesis although the author is aware of its parallel influence on the overall stress response.

#### **4.2.2 Glucocorticoid receptors**

GR and MR are closely related intracellular CORT receptors differing in their ability to attract CORT and in the cellular effects they transduce (Arriza et al. 1987;Lim-Tio et al. 1997). Due to CORT's 10 fold higher affinity for the MR, the receptor is occupied already at basal levels. As CORT has lower affinity for GR, this receptor becomes occupied with increasing CORT levels, such as the circadian elevation prior to the activity period and in response to stress (Reul and De Kloet 1985). In the rat brain, GRs are widely expressed including regions such as amygdala, hippocampus, PVN and pituitary whereas MRs have a more limited distribution but is also highly expressed in the hippocampus and other limbic structures (Reul and de Kloet 1985;Derijk and De Kloet 2008;Cottrell and Seckl 2009).

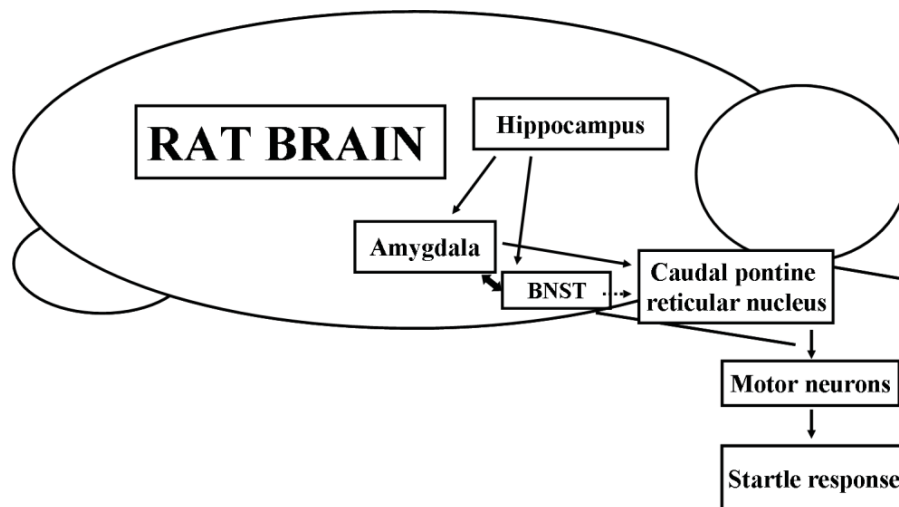
#### **4.2.3 Hippocampus and the HPA axis**

Situated in the central nervous system, the hippocampal formation (hereafter referred to as the hippocampus) is part of the limbic area and commonly considered to include the hippocampus proper, the dentate gyrus, and the subiculum (Jacobson and Sapolsky 1991). The hippocampus is a key regulator of the stress response (Jacobson and Sapolsky 1991;Meaney et al. 1996). Hippocampal neurons exert a tonic inhibitory control of the HPA axis by acting directly on the PVN (Sapolsky et al. 1984) (Fig 1) and/or indirectly via neurons close to the PVN regulating CRH and AVP release (Herman et al. 2002). The hippocampus contains CRH receptors (CRHR1 and CRHR2) and a peptide called corticotrophin releasing hormone binding protein (CRHBP), an endogenous modulator of the biological activity of CRH (Potter et al. 1992). CRHBP has been shown to bind free CRH (Linton et al. 1990) but whether this reduces or stimulates CRH signalling is currently not clear. Through CRH signalling, the hippocampus region can influence other brain areas, including the amygdala (2007) and the bed nucleus of the stria terminalis (BNST) which receives efferents from the hippocampus and subiculum (Swanson and Cowan 1977;Jacobson and Sapolsky 1991) (Fig 2). Communication between these brain areas might be particularly relevant for the ASR expressions discussed in this thesis.

#### **4.2.4 BNST**

The BNST is structurally related to the central amygdala (Alheid et al. 1998). Projections from this area innervate, among many others, the central and posterior

basolateral amygdalar nuclei and hypothalamic paraventricular nucleus (Dong et al. 2001). Thus, the BNST has been implicated in mediation of stress and anxiety-related behaviours in rats (Bangasser and Shors 2008; Lee et al. 2008; Walker et al. 2009). The BNST has been hypothesized to mediate slower-onset, longer-lasting responses, which frequently accompany sustained unspecific threats and persist even after threat termination (Walker et al. 2003; Walker et al. 2009). The brain region is glucocorticoid-sensitive (Lechner and Valentino 1999) and BNST CRH receptors have been shown to be involved in anxiety-associated behaviours, including basal startle increases (Lee et al. 2008; Walker et al. 2009).



**Figure 2:** Schematic and highly simplified drawing of the rat brain with a possible scenario for hippocampus, amygdala and bed nucleus of the stria terminalis (BNST) interplay in relation to the acoustic startle response.

### ***4.3 Prenatal stress***

A large number of human and animal studies show a strong association between an adverse fetal environment (e.g. prenatal anxiety in humans and prenatal exposure to DEX injections, cold exposure or psychological stress in rats) and behavioural and emotional development in later life (O'Connor et al. 2002; Van den Bergh et al. 2005; Tazumi et al. 2005; Abe et al. 2007; Nagano et al. 2008).

#### **4.3.1 Prenatal stress in humans**

Maternal mood has been associated with either increased or decreased fetal movement in response to anxiety/stress in the mother (Van den Bergh et al. 2005). High antenatal

anxiety and stress has also been associated with preterm delivery and low birth weight for gestational age (Talge et al. 2007). On a behavioral scale exposure to prenatal stress has been related to fussiness, activity, attentional and behavioral problems and risk of psychiatric disease (O'Connor et al. 2002; Van den Bergh et al. 2005; Talge et al. 2007). Yet, although clinical studies are invaluable, the results have to be controlled for confounders such as non-uniform prenatal and postnatal stress exposures, which is not always easily done (Van den Bergh et al. 2005). A high degree of control of environmental parameters is one of the strongest benefits of animal studies.

#### ***4.4 Animal studies***

The use of animal models permits controlled evaluation of genetic and environmental factors contributing to stress related phenomena, but in order to extrapolate from rodents to humans critical methodological consideration must be used. In animals, coping with anxiety and stress cannot be measured directly by asking but has to be inferred from the animals' performance in different behavioral tests or in response to psychopharmaca. Timing of brain development differs between humans and rodents (Meyer et al. 2009). Thus with regard to fetal brain development, the gestational period in rats only covers the first and second trimester of human pregnancy whereas the corresponding third trimester is relatively equivalent to the first postnatal week of rat pups (Clancy et al. 2001; Meyer et al. 2009). The circadian rhythm is opposite in humans and rats with rats being nocturnal. The circadian rhythm influences many parameters, including the fluctuations of CORT, which increases towards the beginning of the activity period for both humans and rats (Koehl et al. 1999; Ouellet-Morin et al. 2009) and has been associated with locomotion (Gorka et al. 1996) and acoustic startle response (ASR) magnitude changes (Frankland and Ralph 1995) in rats. In rodent animal models, there are even options with regard to genetic background: The advantage of using an inbred animal strain, with reduced genetic variation, could, however, be arguably less than being able to compare results obtained in an outbred animal strain, such as Wistar or Sprague Dawley rats, with the genetically diverse human population. Especially when considering that stress is subjective in humans.

#### **4.4.1 Prenatal stress in animals**

To mimic prenatal stress exposure in humans a variety of prenatal manipulations are used in animal studies. Among these, restraint stress (Alonso et al. 1991;Morley-Fletcher et al. 2003;Morley-Fletcher et al. 2004), injections of synthetic stress hormone (DEX) (Shoener et al. 2006;Hauser et al. 2006;Hossain et al. 2008;Hauser et al. 2009) and various forms of chronic stress (Weinstock et al. 1992;White and Birkle 2001;Koenig et al. 2005;Tazumi et al. 2005) are frequently employed. The term prenatal manipulation (PNM) will be used henceforward to cover the different prenatal manipulations to ease the reading of the thesis. Even though the prenatal exposures differ more or less from the human situation, the effects in the offspring bear a striking resemblance to the human data of lower birth weight, altered stress response, and increased emotionality, though the phenotype differs between studies. Increased HPA axis responsivity to stress has been observed in PNM animals in some studies (Weinstock et al. 1992;McCormick et al. 1995b;Koehl et al. 1999;Koenig et al. 2005;Shoener et al. 2006), whereas others have found behavioral changes indicative of anxiety (such as changes in acoustic startle response (ASR)) in spite of unaltered HPA axis activity (Hougaard et al. 2005a;Hougaard et al. 2005b;Kjaer et al. 2010).

Further indications of increased emotionality in PNM animals has been inferred from studies in which PNM animals have been slower to leave their home cage in the home cage emergence test (Van den Hove et al. 2005) or enter the open field (Bowman et al. 2004), have shown fewer center entries in the open field test (Weinstock et al. 1992;Abe et al. 2007) or fewer entries into the open arms of the elevated plus maze (Zagron and Weinstock 2006).

#### ***4.5 The unfixedness of fixed genetics – Epigenetics***

Epigenetics refer to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence but can regulate the transcription of the genome (Zhang and Meaney 2010). Studies with rodent models suggest that during both early development and in adult life, environmental signals can activate intracellular pathways that directly remodel the "epigenome," leading to changes in gene expression and neural function (Zhang and Meaney 2010). Epigenetic changes might thus, at least

partly, explain how pre and postnatal stressful manipulations can create long term behavioral changes through glucocorticoid programming or methylation changes.

#### **4.5.1 Glucocorticoid programming**

Programming reflects the action of a factor during a sensitive developmental period or 'window' to affect the development and organisation of specific tissues that are concurrently vulnerable, producing effects that persist throughout life (Seckl 2004). Glucocorticoids are crucial during fetal development for the maturation of tissues and organs and promotion of cellular differentiation (Cottrell and Seckl 2009) but excess amounts of glucocorticoids during prenatal life has been associated with reduced birth weight, HPA axis perturbations and affective disorders in later life (Seckl 2004). It has been suggested that DEX administration in the last week of rat gestation might increase 5-hydroxytryptamine (5-HT) reuptake in the raphe'-hippocampal innervation (Seckl 2001). As 5-HT is key to the maintenance of GR and MR receptor, an increased re-uptake would therefore decrease hippocampal GR and MR expression in foetal and adult hippocampal neurons, but upregulate amygdalar GR expression resulting in an overall increase of HPA axis activity (Seckl 2001). But GR expression could also be altered by methylation changes.

#### **4.5.2 Methylation**

In short, methylation refers to the modification of DNA by methyltransferases that transfer the methyl group from a methyl donor to the gene in question, hereby altering the gene structure and silencing it (Meaney et al. 2007). Environmental events might alter DNA methylation and affect GR gene expression, as an example. Methylation of specific GR promoter regions can reduce GR transcription (Talge et al. 2007). A study by (Weaver et al. 2004) showed an association between maternal care and methylation changes in a hippocampal GR promoter irrespective of the genetic background of the offspring. Thus, offspring of mothers that showed high levels of nurturing was found to have less methylation of the promoter region of the GR gene in the hippocampus, as compared to offspring of low nurturing mothers (Weaver et al. 2004). These differences emerged over the first week of life and could be reversed with cross-fostering (Weaver et al. 2004).

#### ***4.6 Can prenatal stress be adaptive?***

Stressful experiences during gestation and early life has been hypothesized to enhance susceptibility for mental disorders (Maynard et al. 2001;Fumagalli et al. 2007;Cottrell and Seckl 2009). A few studies in both humans and animals have shown an association between mild PNM and protective or adaptive behavioral effects: Mild to moderate levels of prenatal psychological stress were positively associated with mental development and advanced motor development in two year old children (DiPietro et al. 2006) and prenatally stressed rats have shown enhanced learning performance in some studies (Fujioka et al. 2001;Hougaard et al. 2005a). It has yet to be resolved why some individuals thrive under stressful conditions while others strive to survive. But it can be hypothesized that a system, which has been exposed to mild levels of stress during development, might become adapted to handling a stressor postnatally due to epigenetic programming, whereas a naïve system would be less successful. In line with this, differential maternal care in the form of high or low licking and grooming has been shown to yield enhanced contextual learning under contexts of low and high stress, respectively (Champagne et al. 2008).

#### ***4.7 Previous studies***

As mentioned above, the present thesis was based on two previous studies from our lab. Pregnant rats were exposed to CMS during gestation. As adults, their female offspring were tested for reactivity in the ASR test. In the first paper, a persistently increased basal startle was observed in the animals that were stressed during fetal development (Hougaard et al. 2005a). It had earlier been hypothesized that PNM would enhance startle (White and Birkle 2001), but this study was the first to actually show a persistently increased startle response. The study by Hougaard et al. (2005a) differed from previous investigations of PNM and the startle reaction in that these animals had been through a stressful blood sampling procedure 3 months before testing of the startle reaction (Lehmann et al. 2000;White and Birkle 2001;Koenig et al. 2005). Blood sampling was performed under restraint, a procedure that bore some resemblance to startle testing that involves confinement to a test tube. Thus, it was hypothesized that aversiveness associated with blood sampling would lead to enhanced basal startle in PNM animals, due to increased anticipation of aversive stimuli (anxiety) in PNM animals. This hypothesis was supported by results from the subsequent study (Hougaard

et al. 2005b). In this study, we tested two subsets of female offspring. One subset was experimentally naive at the time of startle testing, whereas the other subset had been through a stressful blood sampling procedure, 3 months previously. Chronic stress during fetal development increased the basal startle response in the offspring compared to controls. However, this statistically significant difference was only observed in the subset of PNM animals that had been blood sampled, 3 months before. Interestingly, the startle response was similarly enhanced in offspring of dams, that had been exposed to chronic stress and offspring whose mothers were exposed to DEX.

#### ***4.8 Context***

In the studies by (Hougaard et al. 2005a; Hougaard et al. 2005b)) exaggerated basal startle was only observed in PNM female rats undergoing blood sampling under restraint prior to ASR testing. As ASR testing could be speculated to resemble the experimental circumstances of blood sampling under restraint, this indicated that the PNM animals were more aware of the contextual resemblance between the aversive blood sampling and the ASR test than the control animals. This was supported by a finding of increased capacity for learning in the morris water maze in a subset of the PNM females (Hougaard et al. 2005a). In classic Pavlovian fear conditioning, a conditional stimulus (CS), a specific sound for instance, is paired with an aversive unconditional stimulus (US), an electric shock, in a novel context. After even a single pairing, animals will exhibit fear to the CS (the sound), but also to the other circumstances surrounding the conditioning episode, e.g. conditioning chamber (Anagnostaras et al. 2000). In contrast to conditioned fear stimuli, which are precise time dependant predictors of imminent threat, cues based on contextual circumstances are unpredictable continuous reminders of danger with no warning of time of occurrence, leaving the organism in a sustained state of anxiety (Grillon 2008). Context conditioning can be tested in rats by pairing a context with a shock or otherwise aversive event. After the pairing, the rat will show a fear reaction in that context but not in a novel one (Davis et al. 2010).

## ***4.9 Prenatal manipulations***

### **4.9.1. Chronic mild stress**

A key element of the CMS model is variability and unpredictability to avoid habituation to stressors in the animal (Ottenweller 2000). This was originally implemented in a model by Katz using acute stress factors such as electric shock, tail pinching, and shaking in a random sequence (Katz et al. 1981). To more closely relate to the human situation, the CMS model was developed by Willner et al. based on the work by Katz (Willner et al. 1987). The model consists of a range of milder stress factors (wet bedding, crowding, short lasting food and water deprivation) but keeps the variability and unpredictability in the order and timing of the stressors. CMS was originally developed for use in adult animals to induce depression. Behavioral symptoms often associated with long term use of CMS in adult animals are reduced intake of sucrose or saccharin intake, decrease in self-care, increased latency to enter the first period of rapid eye movement and decreased mobility in the FST (reviewed in (Willner 2005)). When used prenatally, maternal exposure to variable stress regimes has been associated with higher basal startle to the initial pulse (White and Birkle 2001) or more consistently increased basal startle (Hougaard et al. 2005a; Hougaard et al. 2005b) and diminished PPI (Koenig et al. 2005) in PNM male offspring. The latter study did, however, include restraint which has been classified as a severe stressor (Willner 2005). CMS was used as PNM in papers I and IV.

### **4.9.2 Dexamethasone**

The synthetic glucocorticoid DEX is a valuable tool to study effects of glucocorticoids. DEX is a chemical analogue to CORT and can mimic the effects of the stress hormone. In both humans and rodents the placenta has an inbuilt barrier against maternal glucocorticoids in the form of  $11\beta$ -hydroxysteroiddehydrogenase enzymes which inactivate most of the maternal CORT before it reaches the foetus. However, as the enzyme has low affinity for DEX, the synthetic glucocorticoid readily crosses the placental barrier (Seckl 2001). DEX is used in obstetric practice to delay preterm labour and accelerate fetal lung maturation but although consensus exists on the safety and efficacy of a single course of antenatal steroids, knowledge is lacking on the long term developmental consequences of repeated prenatal courses in humans (Lamer 2002; Crowther and Harding 2007). DEX was used as PNM in papers II and III.

## ***4.10 Postnatal manipulations***

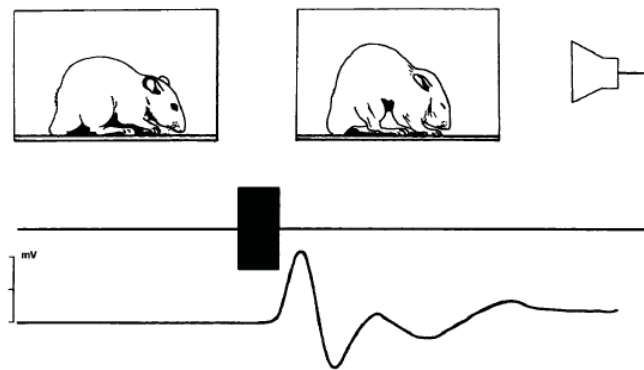
### **4.10.1 Forced swim test**

The FST is used to test the animals for immobility when placed in a tank with water, from which there is no escape. Immobility is interpreted as either failure of persistence in escape-directed behavior (i.e. behavioral despair) or development of passive behavior that disengages the animal from active forms of coping with stressful stimuli (Lucki 1997). The FST is often referred to as a model of depression. This is based on findings that show that if antidepressant treatments are given between the two exposures, the subjects will actively persist in engaging in escape directed behaviors for longer periods of time than after vehicle treatment (Cryan et al. 2002). Decreased mobility has been observed in prenatally restraint stressed offspring (Alonso et al. 1991; Morley-Fletcher et al. 2003; Morley-Fletcher et al. 2004) and in offspring exposed to prenatal psychological stress (Abe et al. 2007). Others have found no association between prenatal restraint stress and decreased mobility (Van den Hove et al. 2005). In order to distinguish between nonspecific differences in activity (locomotive drive) and forced swim specific immobility, the FST is often coupled with the open field test. PNM has been shown to reduce ambulation in some studies (Lehmann et al. 2000; Welberg et al. 2001; Abe et al. 2007) though not in others (Van den Hove et al. 2005; Tazumi et al. 2005; Hauser et al. 2009). When the locomotive behavior as such appears unaffected by treatment, this indicates that the mobility changes in the FST tests is a coping response more than an expression of locomotive drive of the animals.

### **4.10.2 Acoustic startle response**

The ASR is a cross-species characteristic sequential contraction of the skeletal musculature evoked by a sudden and intense acoustic stimulus (Koch 1999) (Fig. 3) with the likely purpose of facilitating flight and to protect the body from a sudden attack (Grillon 2008). Although reflex-like, the magnitude of the response is highly plastic: administration of anxiogenic compounds, such as CRH, increases startle (Swerdlow et al. 1986; Davis et al. 1997), while sensory input such as prepulse inhibition (PPI) can reduce startle (Fendt et al. 2001; Bosch and Schmid 2008). Anticipation of aversive conditions also increases startle. This phenomenon is used in the cue specific fear-potentiated startle procedure and in dark (humans) or light (rats) enhanced startle, which are tests for more generalized anxiety (Davis et al. 2010).

Startle presents several advantages as a tool for investigating fear and anxiety. The similarities of experiments that can be conducted in humans and rodents allow for generalizability of the results. Unlike psychophysiological measures of emotion, such as heart rate and skin conductance, startle increases are typically sensitive to the degree of emotional increase and not simply a result of arousal (Lang et al. 2000;Risbrough and Stein 2006;Davis et al. 2010).



**Figure 3: Modified from (Koch 1999). The acoustic startle response in a rat after stimulus onset. The trace at the bottom of the figure shows the ballistogram of the whole-body ASR. The ASR is usually expressed as arbitrary units or in millivolts (mV) of the accelerometer output which measures the movement of the animal.**

#### 4.10.2.1 Basal startle

Based on a very short latency period from the presentation of the startle eliciting stimulus to the first detectable movement of the body (5 ms in the neck region of rats), the neural circuit behind the startle response must consist of a limited number of synapses (Pellet 1990). Thus, the primary acoustic startle pathway begins with integration of acoustic stimuli in the cochlear root neurons, from which projections transfer the signal to giant neurons in the caudal pontine reticular nucleus (PnC) (Larrauri and Schmajuk 2006). From the PnC, the signal is transmitted to motor neurons leading to activation of skeletal musculature in the face and body which results in the characteristic startle response (Larrauri and Schmajuk 2006)(Fig 4).

The startle response is one of the most widely studied phenotypes in model organisms of relevance to anxiety (Risbrough and Stein 2006). It is modulated by cortical and limbic brain regions, such as the amygdala and BNST (Davis et al. 1997;Swerdlow et

al. 2001;Risbrough and Stein 2006), of which some are abnormally activated in anxiety disorders (Schneider et al. 1999;Lorberbaum et al. 2004). The startle reflex is highly sensitive to fear and anxiety in humans and animals. In humans, elevated startle magnitude is a marker for anxiety disorders, and possibly for major depressive disorder (Grillon 2002;Grillon et al. 2005). In animal studies, various forms of PNM have been associated with increased basal startle in the adult offspring (mild stress combinations (White and Birkle 2001;Hougaard et al. 2005a;Hougaard et al. 2005b) and DEX (Hougaard et al. 2005b;Hossain et al. 2008).

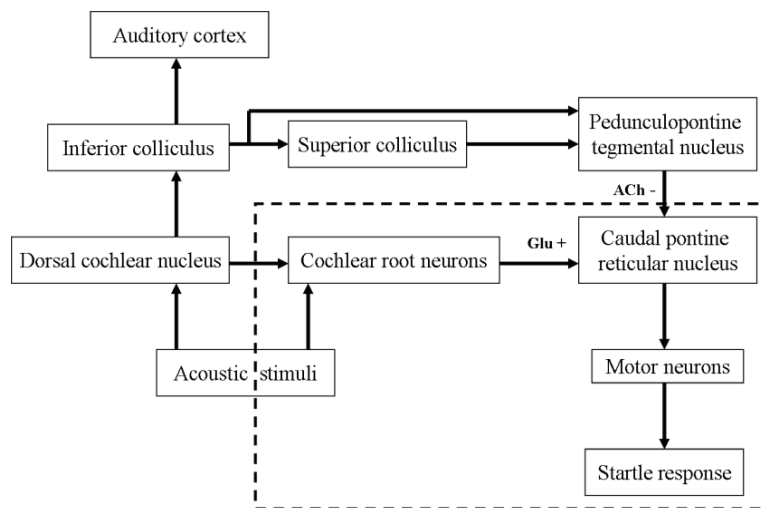
#### **4.10.2.2 Prepulse inhibition**

The basal startle reaction may be decreased when a non-startling (acoustic, tactile or visual) stimulus is presented 20-1000 ms prior to the startle evoking stimulus (Bosch and Schmid 2008). This phenomenon of inhibition is termed PPI and reflects an operational measure of the sensorimotor gating system (Braff et al., 1992). PPI has been proposed to be mediated by a slow inhibitory pathway that runs parallel to the fast excitatory startle pathway (Bosch and Schmid 2008). Experimental data results suggest that the inhibitory pathway originates in the cochlear nuclei which project signals to the inferior and superior colliculus (Larrauri and Schmajuk 2006;Bosch and Schmid 2008). From here neural signals travel to the pedunculo pontine tegmental nucleus (PPT) before interacting with the primary startle pathway on the PnC, possibly through cholinergic projections (Larrauri and Schmajuk 2006;Bosch and Schmid 2008) (Fig 4). Importantly, PPI is not an unconditional response but can be modulated by higher order cognitive processes (attention and learning) through the PPT onto the PnC (Steckler et al. 1994;Larrauri and Schmajuk 2006;Li et al. 2009).

PPI has been hypothesized to allow time for perceptual processing after the stimulus (Graham F 1975;Koch and Schnitzler 1997;Fendt et al. 2001). Increased PPI could therefore be a sign of stronger inhibition on the startle pathway through the PNC. Decreased PPI, on the other hand, has been suggested to represent a protective response; thus, if the prepulse is recognized as novel during processing in the forebrain, inhibition of the PPI occurs, which subsequently allows the animal to startle to protect itself (Larrauri and Schmajuk 2006). PPI may therefore represent a mechanism that allows the

animal to process information before reacting, and under appropriate circumstances, the inhibitory effect of the prepulse may be reduced (Larrauri and Schmajuk 2006).

Decreased PPI have been reported to be associated with a range of neuropsychiatric disorders, including schizophrenia (Braff et al. 1992; Minassian et al. 2007; Bosch and Schmid 2008) and anxiety (Franklin et al., 2009; Ludewig et al., 2002). In animal studies, PPI alterations in the form of either increased (Lehmann et al. 2000; Hauser et al. 2006) or decreased (Koenig et al. 2005) PPI have been observed in PNM animals.



**Figure 4: Schematic outline of the mediating circuit of the basal startle (within the dotted line) and prepulse inhibition of startle (outside the dotted line) circuit. Modified from (Larrauri and Schmajuk 2006). Glu= glutamate; ACh= Acetylcholine. Glu and ACh are neurotransmitters that mediate excitatory or inhibitory signals between brain areas.**

#### 4.10.3 Diurnal phase

The circadian rhythm influences ASR magnitude with up to twofold greater ASR amplitudes during the dark phase (Chabot and Taylor 1992; Frankland and Ralph 1995). If the basal startle response in rats is below maximal expression during the light phase, then testing during this period could be hypothesized to better allow for observation of increased basal startle in PNM animals compared to CONs. Furthermore, stress load might be increased in susceptible animals as i) rats are nocturnal animals and light seems aversive to them, hence the phenomenon of light enhanced startle (Walker and

Davis 1997;Tazumi et al. 2005), and ii) PNM rats, especially females, are more prone to anxiety (Weinstock 2007).

#### ***4.11 Choice of gender***

Female rats were used in the majority of studies in this thesis to allow comparisons with our previous studies (Hougaard et al., 2005a; Hougaard et al., 2005b). We originally chose to use the female rats as epidemiological data have shown that women have higher overall prevalence rates for anxiety disorders than men (Pigott 2003). In animal studies, PNM has been found to induce higher levels of CORT during the light phase in both males and females, but hypercorticism over the entire light/dark cycle in females only (Koehl et al. 1999). Other studies have shown the HPA axis to be selectively altered in PNM female rats (Weinstock et al. 1992;McCormick et al. 1995b;Bakker et al. 1998;Koehl et al. 1999). PNM females have presented more anxiogenic behavior compared with males in the elevated plus maze (Zagron and Weinstock 2006), and open field test (Lehmann et al. 2000;Bowman et al. 2004). But a few studies have suggested that females are less anxious (Nishio et al. 2001;Darnaudery and Maccari 2008). Only a few PNM studies have tested ASR in adult offspring of both genders; two studies found a selective effect on males of either increased startle (Hossain et al. 2008) or increased PPI (though only in one out of two replications) (Hauser et al. 2006) and two studies observed no gender difference (Lehmann et al. 2000;(Burton et al. 2006). More studies are needed to draw any conclusions on selective gender susceptibility with regard to PNM and ASR.

## **5. Objectives of this PhD**

The overall objective of this PhD was to further describe and investigate the animal model of combined pre- and postnatal manipulations developed in the laboratory at the National Research Centre of the Working Environment. Thus, my aim was to investigate the individual components of prenatal and postnatal exposures, and the association between them, which had led to the phenotype of changed reactivity in ASR previously observed in this model (Hougaard et al. 2005a; Hougaard et al. 2005b). This was done by way of a series of conceptual replications of the original studies.

The specific aims of the individual studies were to investigate

1. If the procedure of blood sampling under restraint induced long-term hyperactivity in the HPA-axis in PNM female offspring that could be hypothesized to alter the ASR of PNM rats (paper I).
2. If observation of increased basal startle in PNM animals depended on the phase of testing during the diurnal cycle (paper II).
3. If hippocampal GR and MR expression were associated with increased basal startle (paper II).
4. If prior exposure to a postnatal stressor, without contextual similarities with the ASR test, would induce changes in ASR in PNM animals (paper III).
5. If prenatal exposure to a high versus a lower dose of DEX would result in different behavioral phenotypes with regard to FST and ASR (paper III)
6. If male PNM offspring would present similar ASR changes as observed in female siblings from paper I (paper IV)

## 6. Materials and methods

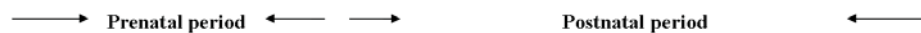
### 6.1 Experiment overview

- Paper I: Female offspring were exposed to CMS during gestation and postnatal blood sampling under restraint prior to ASR testing. After the ASR test, the exposure to blood sampling under restraint was repeated for analysis of plasma CORT.
- Paper II: Female offspring were exposed to 0.2 mg DEX/kg during gestation and postnatal blood sampling prior to ASR testing. The first startle test was performed during the light part of the diurnal cycle. The circadian cycle was reversed 3 weeks prior to the second startle test, which was conducted during the dark part of the diurnal cycle. Hippocampi were dissected from the animals after the behavioral testing and used for western blot and Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) analysis.
- Paper III: Female offspring were exposed to 0.05 or 0.15 mg DEX/kg during gestation and to postnatal FST prior to ASR testing.
- Paper IV: Male offspring (siblings to the females in paper I) were exposed to CMS during gestation and postnatal blood sampling under restraint prior to ASR testing. One month after the ASR testing, the animals were tested in the light enhanced startle test.

**Table 1: Experiment overview**

Study	Rat strain	PNM	DEX dose mg/kg	GD	Gender	BS 3-5 m.	FST 3 m.	ASR 6-7 m.	CORT	Protein GR	mRNA GR, MR, CRHBP CRHR1 CRHR2
Study I	Wistar	CMS		9-21	F	X, X*		X	X, X		
Study II	Sprague Dawley	DEX	0.2	14-21	F	X		X		X	X
Study III	Wistar	DEX	0.15/ 0.05	14-21	F		X	X			
Study IV	Wistar	CMS		9-21	M	X		X	X		

\* at 8 m.



**PNM: Prenatal manipulation; CMS: Chronic mild stress; DEX: Dexamethasone; GD: Gestational day; BS: Blood sampling under restraint; FST: Forced swim test; ASR: Acoustic startle response; F: female; M: Male; CORT: Corticosterone; GR: Glucocorticoid receptor; CRHBP: Corticotrophin releasing hormone binding protein; CRHR1 and2: Corticotrophin releasing**

**hormone receptor 1 and 2; MR: Mineralcorticoid receptor; mRNA: messenger ribonucleic acid; m.: months.**

## ***6.2 Animals (papers I- IV)***

Wistar or Sprague Dawley rats (Table 1) were obtained from HanTaC:WH, SPF or Taconic M&B, Denmark, respectively. The animals were housed under controlled environmental conditions with a 12-h light–dark cycle. Chow pellets and tap water were provided ad libitum. Clean cages and new bedding were provided once or twice weekly. The animal welfare committee, appointed by the Danish Ministry of Justice, granted ethical permission for the studies. All procedures complied with the EC Directive 86/609/EEC and with the Danish law regulating experiments on animals.

## ***6.3 Prenatal manipulations***

### **6.3.1 CMS (papers I and IV)**

Pregnant rats allocated to CMS were exposed from GD 9-21 in line with Hougaard et al (2005a,b). Each stressor was applied once or twice during the period of exposure. Stressors were distributed over the 24-h cycle, and stressors that were used twice were separated by at least 3 days. The individual stressors consisted of: being housed in a mouse cage (25x20x14 cm; 1x8h, 1x16h); food and water deprivation (2x8h); empty water bottle (1 h); wet bedding (1x9h, 1x16h); crowding (2x8h); new partner (1x24h); isolation (1x16h); tilted cage (45°; 1x8h, 1x16h) and wire cage (1x8h, 1x16h).

### **6.3.2 Dexamethasone (papers II and III)**

DEX (Sigma-Aldrich, Denmark) was dissolved in 4% ethanol/isotonic saline. Pregnant rats allocated to the DEX groups were injected subcutaneously once daily, between 9 and 12 am from GD 14 to GD 21.

## ***6.4 Postnatal manipulations***

### **6.4.1 Blood sampling under restraint (papers I, II and IV)**

At some point from 3-8 months of age, depending on the respective study, offspring were exposed to blood sampling under restraint. Briefly, each animal was restrained in an immobilizer, and blood was collected from a vertical incision in one of the tail veins (basal sample). After an additional 20 min of restraint, blood was collected again (stress sample). The rat was subsequently returned to its cage, and one hour later brought back to the laboratory, restrained and sampled for the post stress sample.

### 6.4.2 Forced swim test (paper III)

Female offspring allocated to the FST test were tested twice, 24 hours apart. This reveals the effect of repeated exposure to the swim test in addition to the novel experience of the first time. The individual animals were placed in a transparent cylindrical tank made from acrylic plastic (H: 55 cm, D: 24 cm) (Fig. 5A). Each tank contained 38 cm of tap water (25° C) which was changed between each trial. On day 1 (training phase), each rat was in the tank for 15 min, and on day 2 (test phase) for 5 min. Animals were evaluated for 5 min in total on both test days, with a behavioural score for the dominant activity in 5 second intervals.

Activity was classified as 1) Struggling: High activity level. The animal was almost upright in the water while paddling on the walls of the tank, e.g. the front legs broke the water surface. The surface of the water was clearly disturbed. Diving was included as struggling as it was often followed by paddling. 2) Swimming: Intermediate activity level. The animal displayed searching behaviour, e.g. moved along the rim of the tank as if looking for an exit. The body of the animal was fairly horizontal. The animal could be observed to shift position in the tank. 3) Immobility: Low activity level. The animal only made the necessary movements to keep afloat. The water surface was very calm (Fig. 5B).



Figure 5: A) Laboratory set-up for the forced swim test, B) Immobile rat in tank during forced swim test.

### 6.3 ASR test (papers I-IV)

All the offspring were tested for ASR, at 6-7 months of age, using SR-Lab™ SDI startle response system (SanDiego Instruments INC.,USA). Testing was conducted as

previously described (Hougaard et al. 2005a). Throughout the startle protocol, white background noise (70 dB(A)) was delivered continuously inside the chambers. A 5 min acclimatization period commenced test sessions. The startle eliciting stimulus consisted of a 40 ms broadband 120 dB(A) noise-burst. Each session started and ended with 5 120 dB(A) startle trials followed by 35 test trials delivered in semi-randomized order (10 startle trials of 120dB(A); 5 each of 4 prepulses (72, 74, 78 and 86 dB(A), respectively) + startle trials (denoted PPI72, PPI74, PPI78, and PPI86, respectively); 5 trials with no stimulus except background noise). Movement of the tube was registered for 100 ms after onset of the startle stimulus (sampling frequency 1k Hz), amplified, and the average response over 100 ms (AVG) was calculated. For each level of prepulse, AVGs were averaged and used for calculation of PPI. PPI was expressed as percent reduction in AVG compared to the average of the 10 middle startle trials:  $\%PPI = 100 - ((AVG \text{ at prepulse} + \text{startle-trial}) / (AVG \text{ at startle trial}) * 100\%)$ .

#### ***6.4 Biochemical and molecular analyses***

##### **6.4.1 Corticosterone in blood samples by radioimmunoassay (papers I and IV)**

CORT in plasma was determined by competitive radioimmunoassay as previously described (Pedersen et al., 2000)<sup>2</sup>. The samples were analyzed in duplicate. Plasma was prepared as previously described and was diluted in PBS (pH 7.4) and stored at -80°C.

##### **6.4.2 Preparation of hippocampi samples (paper II)**

Rats were collected from the animal room one by one and decapitated immediately (Harvard 55-0012 Small Animal decapitator, Scanbur AB, Sweden) upon being brought into the laboratory. The brain was transferred immediately onto a cooled plate and divided along the midsagittal line. One hemisphere was frozen directly in pulverized dry ice and the hippocampus from the other half was isolated and frozen directly before being transferred to Eppendorf PCR tubes and stored at -80 °C. As the different hemispheres (left (L) or right (R)) of the hippocampus were analyzed separately, this resulted in four subgroups (CONL, DEXL, CONR and DEXR).

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<sup>2</sup> This procedure was performed by Ulla Tegner at the National Research Centre for the Working Environment.

### **6.4.3 Tissue preparation for western blotting (paper II)**

Frozen hippocampi were mixed with cold homogenization buffer (20 mL/g tissue) and 1% protease inhibitor (Sigma-Aldrich, USA). After homogenization, the tissue was centrifuged at 3600 rpm for 10 min at 4°C (Fischer Scientific Accuspin™ Micro R, CA), yielding supernatant and pellet. Isolation and centrifugation of the resulting supernatant at 10800 rpm for 15 min yielded the cytosolic fraction. The nuclear fraction was isolated by resuspending the pellet in 200-400 µL homogenization buffer followed by sonication (Misonix, XL-2000, Qsonica, LLC. Newton, CT, USA)(2 x 10 seconds bursts, step 3) and ultracentrifugation (Beckman Coulter Optima™ L-90K, Fullerton, CA) at 18000 rpm, 20 min at 4°C. The final supernatant comprised the nuclear proteins.

### **6.4.4 Western blotting (paper II)**

The protein concentration in the individual samples was determined by protein assay. Samples (20 µg) were mixed with 25% Laemmli loading buffer and loaded onto bis-acrylamide gels and the proteins separated by SDS polyacrylamide electrophoresis before being transferred to membrane. All blocking and incubation with antibodies steps listed were done with a 5% milk powder 1xPhosphate Buffered Saline (PBS) solution (pH 7.4) solution. The antibodies used in the western blotting were monoclonal Anti-β-Actin mouse antibody (Sigma-Aldrich Inc, USA) (1:2000) as a control for the loaded amount of protein; BuGR2 (1:500 in 5% milk powder, 1xPBS solution) and secondary anti-mouse IgG Peroxidase (Sigma-Aldrich Inc, USA) either in a (1:20000) or (1:10000) concentration. Immunopositive bands were visualized using the Enhanced chemiluminescence (ECL) plus western blotting system from (Amersham, UK). All samples were run in duplicate.

### **6.4.5 RNA extraction (paper II)**

Hippocampi from 4 non-sibling CON and 3 non-sibling DEX animals were used for the RT-qPCR analysis<sup>3</sup>. Tissue homogenization, RNA extraction, RNA characterization, cDNA synthesis, and RT-qPCR were carried out as previously described by (Elfving et al. 2008). Briefly, hippocampi were homogenized in Lysis buffer and total RNA was isolated. Aliquots of the RNA solution were taken for both RNA quantification and qualification. The integrity of RNA and the RNA concentration were determined with

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<sup>3</sup> All the RT-qPCR work was performed by Betina Elfving and Pia Høgh Ploughman at Centre for Psychiatric Research, Aarhus University Hospital.

RNA StdSens microfluidic chips. The RNA purity and the RNA concentration were determined by spectrophotometer (UV1650PC Shimadzu, Kyoto, Japan). Data on quality and purity of the extracted RNA were evaluated with student's t-test. Afterwards RNA was reversely transcribed using random primers and Superscript III Reverse Transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. The cDNA samples were diluted 1:30 with DEPC water before used as a qPCR template

#### **6.4.6 Real-time quantitative polymerase chain reaction (paper II)**

The RT-qPCR reactions were carried out in 96-well PCR-plates using the Mx3000P (Stratagene, USA) and SYBR Green. Each SYBR Green reaction (10 µl total volume) contained 1x SYBR Green master mix (BIORAD, CA, USA), 0.5 µM primer pairs, and 3 µl of diluted cDNA. The gene expression of GR, MR, CRHR1, CRHR2, CRHBP and the two reference genes 18s subunit ribosomal RNA (18s rRNA) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (Ywhaz) were investigated. The primers were designed and tested as described by Elfving et al (2008).

#### **6.4.7 Data analysis**

Data for each individual were normalized with the geometric mean of the Ywhaz and 18s rRNA reference genes. Stability comparison of the expression of the reference genes was conducted with the Normfinder software (<http://www.mdl.dk>) (Andersen et al. 2004).

### **6.5 Statistical procedures**

Papers I and IV: Plasma CORT was analyzed by three-way ANOVA with Group (CON and CMS) and Blood sampling (+/-) as between-subject factors, and Sample (basal, stress, and post-stress) as within-subject factor (repeated measure).

Papers I-IV: Basal startle data were analyzed by two-way ANOVA, with Group (CON and CMS or DEX) and Blood sampling (+/-), FST or circadian phase as factors depending on the study. When appropriate ( $p < 0.05$  in overall ANOVA), Fishers Least Significant Difference Test was applied for protected pair wise comparisons. For PPI, data were analyzed separately for each level of prepulse. Also when appropriate, pups were nested within their litters to avoid litter effect.

Study II: Western blot data were analyzed by one-way ANOVA with each group (CON or DEX) divided in separate subgroups (CONL, DEXL, CONR and DEXR). Real time qPCR data were analyzed with t-test.

For all the studies, the accepted level of statistical significance was  $<0.05$  (SYSTAT Software Package version 12).

## 7. Results

### 7.1 Result overview

Table 2 gives an overview of the behavioral results observed in our model in the studies conducted during my PhD. In short, the studies presented the following results:

- Paper I: Female offspring, exposed to CMS during gestation, showed decreased PPI compared with CON animals. Plasma CORT levels, basal and in response to restraint, were similar in CON and PNM animals both prior to and after ASR testing. The blood sampling procedure was associated with increased PPI at the two highest prepulse intensities irrespective of prenatal exposure.
- Paper II: Female offspring exposed to 0.2 mg DEX/kg during gestation and blood sampling under restraint as adults, showed increased basal startle during the light phase compared with CON animals. Basal startle also seemed increased in DEX compared to CON offspring in the dark phase, though this difference only tended to attain statistical significance. No difference was found between DEX and CON animals for hippocampal GR, MR, CRHR1 and 2 expression levels. CRHBP tended towards increased expression in DEX animals.
- Paper III: Female offspring exposed to 0.15 mg DEX/kg during gestation showed decreased mobility in the FST test. ASR was similar in the DEX and CON animals.
- Paper IV: Male offspring, exposed to CMS during gestation, presented similar ASR alterations to the females in paper I in that PPI was decreased in non-sampled CMS males and increased in males exposed to prior blood sampling under restraint.

**Table 2: Behavioral results**

Study	Rat strain	PNM	Dose mg/kg	Gender	BS	FST 3 m.	ASR 6-7 m.
Study I	Wistar	CMS		F	No diff.		↓ PPI in PNM animals ↑ PPI in animals exposed to BS
Study II	Sprague Dawley	DEX	0.2	F			↑ Basal startle in PNM animals
Study III	Wistar	DEX	0.15 / 0.05	F		↓ mobility in DEX 0.15 group	no difference
Study IV	Wistar	CMS		M	No diff.		↓ PPI in PNM animals ↑ PPI in PNM animals exposed to BS

→ Prenatal period ←      → Postnatal period ←

**PNM: Prenatal manipulation; CMS: Chronic mild stress; DEX: Dexamethasone; BS: Blood sampling under restraint; F: female; M: Male; PPI: prepulse inhibition; m.: months.**

### 7.2 *CORT measurements (Papers I and IV)*

Paper I: CORT was measured in plasma from blood sampling under restraint in female offspring exposed to prenatal CMS at 3 and 8 months of age. The concentration of plasma CORT differed statistically significantly between samples at both ages ( $P < 0.001$ ) (Fig 6A&B).

Paper IV: CORT was measured in plasma from blood sampling under restraint in male offspring exposed to prenatal CMS at 3 months of age. The concentration of plasma CORT differed statistically significantly between samples ( $P < 0.001$ ).

No statistically significant difference was related to prenatal CMS in either gender irrespective of time point.

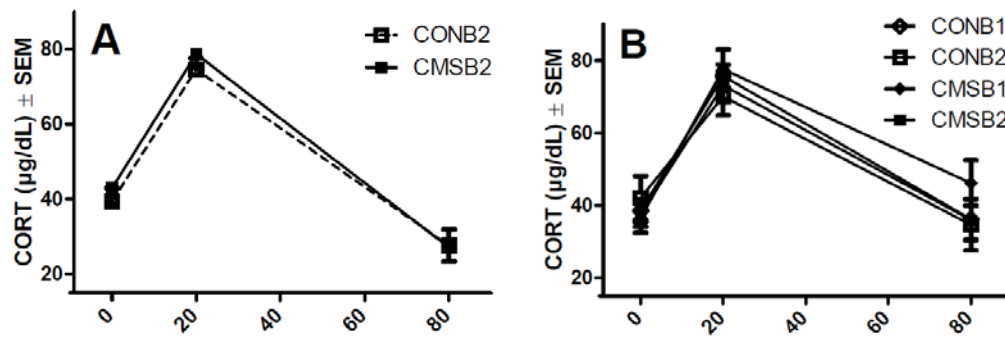
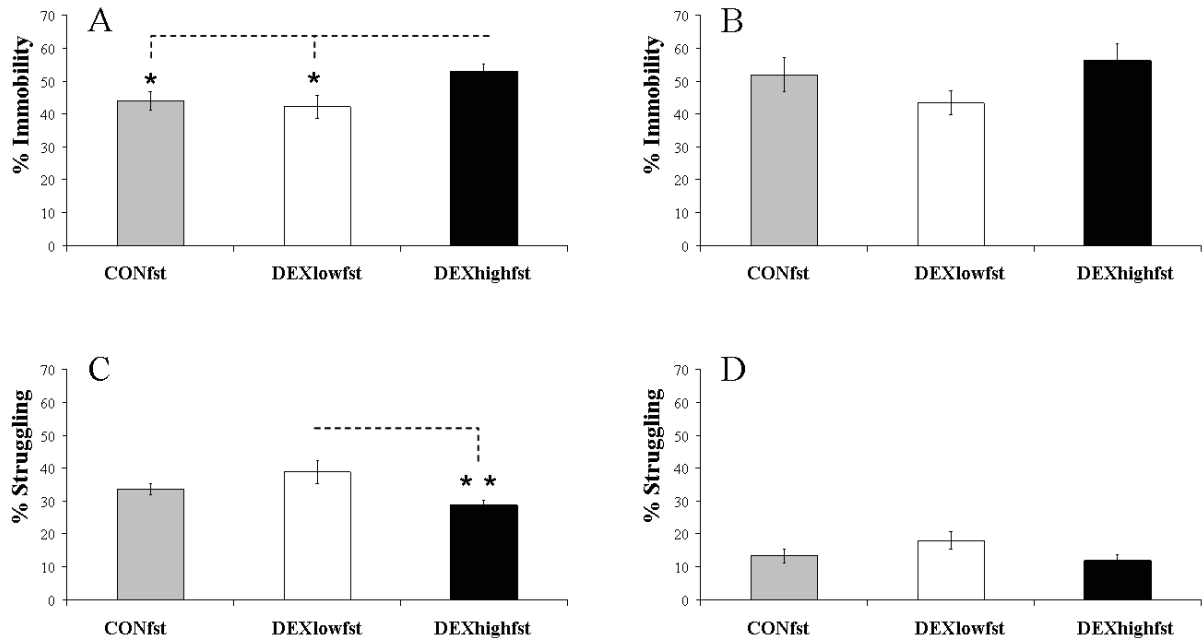


Figure 6: Mean corticosterone response to blood sampling under restraint in female rat offspring at age 3 months A) and 8 months B). CONB1 = control + blood sampled at 8 months. CONB2 = control + blood sampled at both 3 and 8 months. CMSB1 = prenatal stress + blood sampled at 8 months. CMSB2 = prenatal stress + blood sampled at both 3 and 8 months. T0 = 0 min, basal sample; T20 = after 20 min restraint, stress sample; T80 = 60 min after the restraint, post stress sample. Mean  $\pm$  SEM, n=16.

### 7.3 FST Paper III

CON offspring and offspring from two different prenatal DEX exposure groups (DEX<sub>low</sub>, 50 µg/kg; DEX<sub>high</sub>, 150 µg/kg) were tested in the FST, at the age of 3 months, prior to ASR testing. On day 1, the groups showed statistically significant differences in immobility ( $P=0.018$ ) and struggling ( $P=0.013$ ), respectively. Pairwise comparisons regarding effect of prenatal background demonstrated significantly increased immobility in DEX<sub>high</sub> rats compared with CON ( $p=0.021$ ) and DEX<sub>low</sub> ( $P=0.012$ ) (Fig 7A), and significantly less struggling in DEX<sub>high</sub> compared with DEX<sub>low</sub> ( $P=0.003$ ) (fig. 7C). The same behavioural activity was visible on day 2, although not statistically significant (fig. 7B and D).



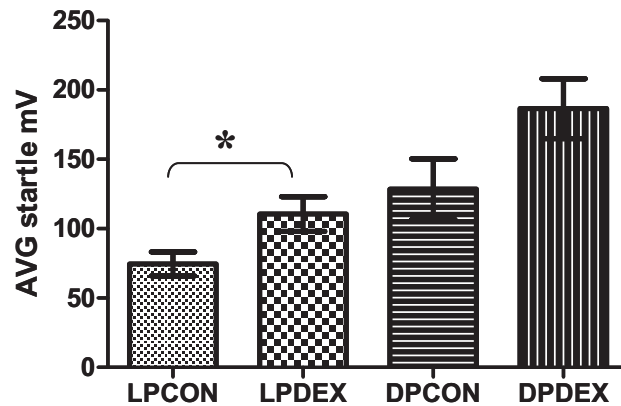
**Figure 7: Forced swim test in female rats at 3 months of age. A) Percent time spent immobile during initial 5 min (day 1), Group [F(2,43)=4.419; p=0.018]. B) Percent time spent immobile during initial 5 min (day 2). C) Percent time spent struggling during initial 5 min (day 1), Group [F(2,43)=4.833; p=0.013]. D) Percent time spent struggling during initial 5 min (day 2) CONfst= control+forced swim test, DEXlowfst= prenatal DEX 50 µg/kg +forced swim test, DEXhighfst= prenatal DEX 150 µg/kg +forced swim test. n= 16, 12, and 18 for CON, DEXlow and DEXhigh, respectively. Mean ± SEM. \* P< 0.05, \*\* P<0.01.**

## 7.4 ASR

### 7.4.1 Basal startle

Paper I: Similar basal startle was observed in CON and PNM (CMS) female offspring irrespective of exposure to postnatal blood sampling under restraint prior to ASR testing.

Paper II: PNM female offspring exposed to DEX during gestation and postnatal blood sampling showed increased basal startle. Overall statistical analysis revealed effect on basal startle of prenatal exposure (DEX: P=0.008) and of circadian phase during testing (Phase: P<0.001) (Fig. 8).



**Figure 8:** Mean basal startle response to the middle 10 120 dB pulses in female offspring tested during the light and dark phase of the circadian cycle with a 3 week acclimation period in between. Testing was performed at age 6-7 months. LPCON = controls tested during light phase, LPDEX= prenatally exposed to Dexamethasone tested during light phase, DPCON = controls tested during dark phase, DPDEX= prenatally exposed to Dexamethasone tested during dark phase. Mean  $\pm$  SEM, n=17-20. \*effect of DEX,  $P<0.05$ .

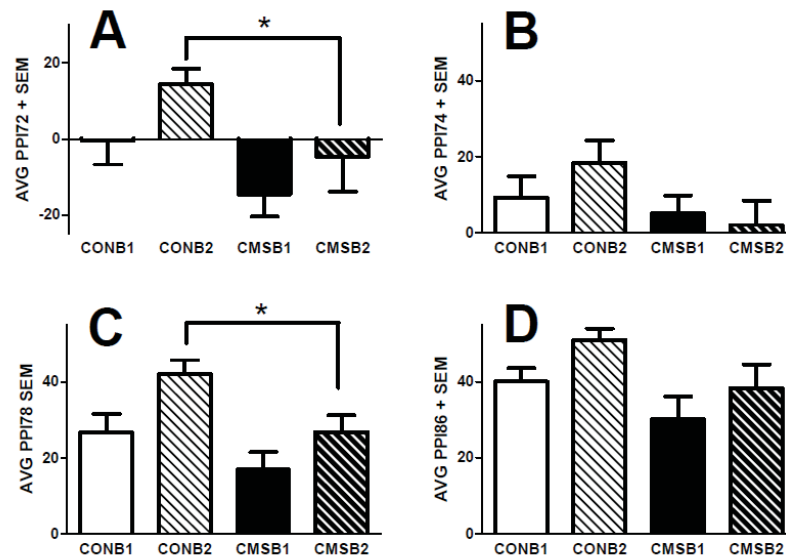
When circadian phases were analyzed separately, basal startle was statistically significantly increased in DEX compared to CON offspring during the light phase during the first test session ( $P=0.021$ ). When tested during the dark phase three weeks later, basal startle also seemed increased in DEX compared to CON offspring, though this difference only tended to be statistically significantly different ( $P=0.07$ ).

Paper III: PNM female offspring, exposed to DEX during gestation, showed similar basal startle irrespective of exposure to FST prior to ASR testing.

Paper IV: Similar basal startle was observed in CON and PNM male offspring, exposed to CMS during gestation, irrespective of exposure to postnatal blood sampling under restraint prior to ASR testing.

#### 7.4.2 PPI

Paper I: Female offspring exposed to prenatal CMS showed decreased PPI. Overall analysis indicated statistically significant differences related to prenatal CMS for PPI72, PPI78, and PPI86 (CMS:  $P=0.012$ ;  $P=0.005$ , and  $P=0.017$ , respectively). Pairwise comparisons showed an association between prenatal CMS and PPI. Blood sampled PNM animals showed decreased PPI compared with blood sampled CON for PPI72 ( $P=0.048$ ) and PPI78 ( $P=0.018$ ) (Fig. 9A and D).



**Figure 9:** Mean average PPI72 (a), PPI74 (b), PPI78 (c) and PPI86 (d) in female offspring at the first week of testing during the dark phase at 6 months of age. CONB1 = control, CONB2 = control + blood sampled at 3 months. AVG = average. PPI = prepulse inhibition. CMS = prenatally stressed with chronic mild stress. CMSB1 = prenatal stress. CMSB2 = prenatal stress + blood sampled at 3 months. General effect of prenatal background on PPI72, PPI78, and PPI86: CMS: [F(1,59) = 6.774; P = 0.012], [F(1,59) = 8.585; P = 0.005], and [F(1,59) = 5.997; P = 0.017], respectively. PPI78 and PPI86 also showed effect of postnatal blood sampling: [F(1,59) = 8.179; P = 0.006] and [F(1,59) = 3.802; P = 0.056], respectively. Mean  $\pm$  SEM, n = 16. \* P < 0.05.

Blood sampling 3 months previously increased PPI at the two highest prepulse intensities irrespective of prenatal exposure, c.f. Fig. 9C and D (Blood Sampling: P = 0.006 and P = 0.056 for PPI78 and PPI86, respectively). Pairwise comparisons regarding the effect of blood sampling showed that PPI was increased in blood sampled compared to non-sampled control offspring at PP78 (CONB1 vs. CONB2: P = 0.017).

Papers II and III: PPI was similar in PNM and CON female offspring irrespective of postnatal exposure to blood sampling or FST, respectively, prior to ASR testing.

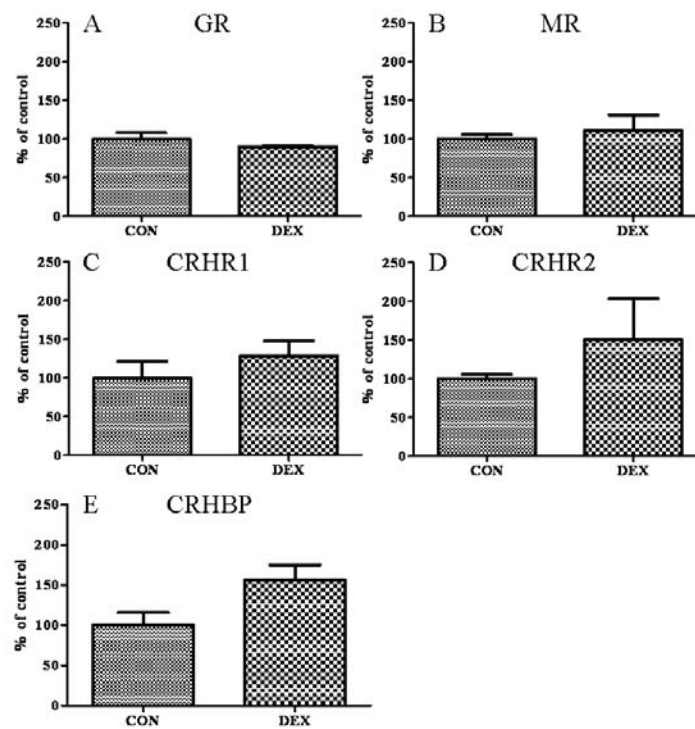
Paper IV: PNM male offspring exposed to CMS during gestation presented an effect of blood sampling at PPI72 (P = 0.015) and PPI74 (P = 0.017). No statistically significant differences were present for control offspring.

### 7.5 Hippocampal protein expression (Paper II)

Quantification of optic density of the GR bands after normalization with  $\beta$ -Actin revealed no statistically significant difference between the 4 groups (CONL, DEXL, CONR, DEXR) ( $P=0.343$ ) indicating that hippocampal GR expression was similar in the prenatal exposure groups and in the brain hemispheres.

### 7.6 Hippocampal mRNA expression (Paper II)

CON and the DEX animals were similar with respect to 18s/28s rRNA ratio or RNA purity (data not shown). The normalized mRNA levels of GR, MR, CRHR1, CRHR2, and CRHBP in hippocampus are given in Fig 10A-E. For the first 4 genes, the mRNA levels were similar in the CON and the DEX animals (Fig. 10A-D). CRHBP showed a strong trend towards increased expression in the DEX animals compared to CON ( $P=0.07$ ) (Fig 10E).



**Figure 10:** Messenger RNA samples from hippocampus of control (CON, n=4) and prenatal dexamethasone (DEX, n=3) groups were used for quantification of the expression levels of glucocorticoid receptor (GR) (A), mineralcorticoid receptor (MR) (B), corticotrophin releasing hormone receptor1 (CRHR1) (C), corticotrophin releasing hormone receptor2 (CRHR2) (D) and corticotrophin releasing hormone binding protein (CRHBP) (E) using real-time qPCR. Values for each individual were normalized with the geometric mean of the reference genes *Ywhaz* and 18s rRNA. Plotted data show mean group values + SEM of mRNA expression as % of control.

## 8. Discussion

Four papers describe our investigations of the influence of pre- and postnatal manipulations in our animal model: Paper I investigated whether chronic hyperactivity in the HPA axis, inferred from elevated CORT levels, as a result of the exposure to blood sampling under restraint, could be hypothesized to alter the ASR of female PNM rats. Paper II investigated whether observation of increased basal startle in PNM female animals depended on the phase of testing during the diurnal cycle, and whether hippocampal GR and MR expression were associated with increased basal startle. Paper III investigated if prior exposure to the FST, a postnatal stressor without contextual similarities with the ASR test, would induce changes in the ASR in PNM female animals and paper IV investigated whether male PNM offspring would show similar ASR changes as observed in female siblings from paper I. In the following, the results of the four papers will be discussed in relation to the aims of the individual studies and in relation to the animal model as such.

### ***8.1 CORT, CRH and ASR (papers I, II and IV)***

No change in CORT levels were observed in PNM animals in our studies despite observations of altered ASR in the same animals in line with the original studies (Hougaard et al. 2005a; Hougaard et al. 2005b; Kjaer et al. 2010; papers I and IV). This indicates that the altered ASR observed, in the form of either increased basal startle or decreased PPI, was not induced by chronic hyperactivity in the HPA axis. However, postnatal administration of CRH has been shown to intensify ASR in rats (Dunn and Berridge 1990; Lee and Davis 1997; Servatius et al. 2005) even after adrenalectomy (Lee et al. 1994). Therefore, the neural circuit regulating the ASR response may involve only some of the components of the HPA axis, e.g. signalling pathways upstream or downstream of CRH. In paper II, increased basal startle in PNM animals showed a slight association with hippocampal CRHBP. Furthermore, CRH is believed to play a special role in long duration cues (Davis et al. 2010), supporting a role for CRH in the long term ASR alterations observed in our animals. The BNST is a key area for CRH-enhanced startle and has been hypothesized to mediate slower-onset, longer-lasting responses, which frequently accompany sustained unspecific threats and persist even after threat termination (Walker et al. 2003). It could be hypothesized that CRH through

the BNST is a possible candidate for mediating the long lasting effects of PNM observed in our studies, explaining why the enhanced startle observed 3 months after the blood sampling may have occurred without alterations in CORT (Hougaard et al. 2005a;Hougaard et al. 2005b).

### ***8.2 Diurnal phase and ASR (paper II)***

Startle response patterns during both the light and the dark diurnal phases were almost similar in paper II. This suggests that the somehow reduced difference during the dark phase could be mediated by habituation to the startle procedure, rather than by a differential effect of the prenatal exposure on startle during the two diurnal phases. A span of three weeks between ASR testing may not suffice to overcome habituation as response decrement, produced by as little as a single auditory stimulus, has been retained in rats without detectable loss for up to 30 days (Leaton 1976). Other studies have conducted the ASR testing of PNM animals during the light phase without observing increased basal startle, which supports our view that the diurnal phase cannot be the sole determinant of increased startle in PNM animals (Koenig et al. 2005;Burton et al. 2006).

### ***8.3 Hippocampal GR and MR expression in PNM animals (paper II)***

Prenatal DEX (Levitt et al. 1996;Welberg et al. 2001) or restraint stress (Szuran et al. 2000) during the last gestational week has been shown to reduce hippocampal GR and/or MR mRNA expression. In paper II, DEX exposure during the last gestational week showed no association with hippocampal GR (mRNA and protein) and MR (mRNA) expression. Interestingly, results from another study indicate that postnatal restraint might interfere with glucocorticoid receptor expression in animals exposed to prenatal DEX (Shoener et al. 2006). MR expression was reduced in prenatal DEX offspring that had not been exposed to postnatal restraint, but similar hippocampal GR and MR mRNA levels were observed in prenatal DEX and CON animals after postnatal restraint stress (Shoener et al. 2006). Based on this study, our rats, when subjected to postnatal restraint, would not be expected to differ in hippocampal GR or MR expression despite prenatal exposure to DEX. These findings indicate that not just prenatal glucocorticoid exposure but also postnatal experiences influence adult GR and MR expression. This could be a demonstration of epigenetic changes which have been

speculated to be reversible. Thus it has been suggested that DNA methylation, although extremely stable, can be altered at different points in life under the right circumstances (Meaney et al. 2007).

Since the expression of these receptors was similar in both PNM and CON animals (paper II), hippocampal expression of these receptors does not appear to be directly involved in the increased startle response in animals exposed to DEX in utero.

#### ***8.4 Blood sampling under restraint or FST (paper III)***

The combination of prenatal CMS or prenatal DEX exposure (100 µg/kg) with postnatal stressful blood sampling was associated with increased basal startle in the two previous studies (Hougaard et al. 2005a; Hougaard et al. 2005b) as well as in paper II. We hypothesized that the PNM rats might conceive a contextual link (limited freedom of movement) between the two experiences which would not be conceived as strongly by CONs. In line with this, Fujioka et al. (2001) reported enhanced ability to remember an association between an aversive stimulus and contextual clues in PNM rats. Increased basal startle can be interpreted as increased levels of anxiety or fear as the startle response can be increased by anticipation of aversive stimuli (Davis et al. 2010). Increased contextual awareness in PNM animals may therefore correspond with observation of anxious patients being sensitive to threatening contexts (Grillon 2002). The combination of prenatal DEX exposure with FST was unassociated with changes in basal startle despite clear indications of affected behavior in DEX<sub>high</sub> rats in the FST (paper III). Lack of contextual clues between the FST and ASR test might be the explanation. If the rats received no contextual warning of specific danger when placed in the ASR apparatus, this could explain why they showed similar basal startle levels to the CON animals. But, since the blood sampling under restraint procedure was not included in this study, it remains speculative whether the contextually more similar stressor would have elicited increased startle in the present experiment, as observed in Hougaard et al 2005a,b and paper II.

It should be noted, that female PNM offspring showed decreased PPI in paper I without having been exposed to blood sampling under restraint. This indicates that PPI changes in PNM animals does not require a triggering event (contextual aversive similarity between blood sampling and ASR) to show in the ASR test.

### ***8.5 PNM, ASR and gender differences (papers I and IV)***

As described in 4.11 Choice of gender animal studies of PNM have shown a tendency towards a differential effect of PNM on gender (McCormick et al. 1995a; Nishio et al. 2001; Weinstock 2007; Darnaudery and Maccari 2008) with PNM females showing a tendency towards more anxiogenic behavior compared with males. We found no gender difference on plasma CORT levels and similar PPI alterations in PNM male and female animals (papers I and IV). We did however, observe a difference between male and female CON animals as blood sampled female CON animals showed increased PPI whereas no difference was observed between sampled and non-sampled male CON animals (papers I and IV). There might be a selective gender sensitivity of the developing brain areas to stress hormones (Weinstock 2007). A tentative hypothesis could be that the female ASR neural circuit is more sensitive to the stressful experience of blood sampling under restraint than males and that exposure to PNM might feminize the males with regard to ASR expression (paper IV).

### ***8.6 Basal startle and PPI alterations in PNM animals – an influence of allostatic load? (papers I-IV)***

Simultaneous PPI and basal startle changes were not observed in any of our studies and we have not been able to locate other PNM studies in rats that have shown simultaneous changes in basal startle and PPI (Lehmann et al. 2000; Koenig et al. 2005; Hougaard et al. 2005a; Hougaard et al. 2005b; Hauser et al. 2006). However, concomitantly enhanced startle and reduced PPI have been observed in male rats after eight weeks of isolation rearing starting at weaning (Du et al. 2010). To understand how the two behavioral functions might be differentially modulated by apparently similar circumstances requires a short introduction to a few concepts: Basal startle may be top-down regulated by more than one neural network, which is exemplified by the distinction between fear-potentiated startle (mediated by short duration cues through the central amygdala) and CRH enhanced startle (mediated by long duration cues through the BNST) (Davis et al. 2010). And a brain area often plays multiple roles. For instance, both startle enhancements probably involve prior signalling from the basolateral amygdala (Davis et al. 2010). The amygdala has also been implicated in modulation of PPI (Du et al., 2010; Li et al., 2009), and the BNST appears to be involved in the development of associative context fear (Walker et al. 2009). In fact, both the hippocampus and the

BNST have been associated with context conditioning (Grillon 2008). This might be important, if the contextual similarities between blood sampling under restraint and ASR testing are indeed influential on the altered ASR observed in our studies.

To return to the original question of how either basal startle or PPI might be modulated without affecting the other, it stems from the fact that our studies have yielded either increased basal startle (Hougaard et al 2005a,b; paper II) or PPI alterations (papers I and IV). It may be hypothesized that the varying effects of PNM on PPI and basal startle observed in our studies can be explained by the existence of a certain threshold in the induction of a stress response in the amygdala and BNST, dictating which brain region is primarily active with regard to ASR modulation. The ASR modulation might be further affected by contextual clues via the hippocampus, though this could depend on the degree of contextual awareness in the animals, e.g. the PNM might be more sensitive to contextual similarities than CON as suggested in 8.4 Blood sampling under restraint or FST (paper III). In the case of conceived contextual similarity between an experience of postnatal manipulation and the ASR test, CRH signaling from the hippocampus might separately influence the PPI (through the amygdala) or basal startle expression (through the BNST) (Fig. 2).

Altogether, the interplay between hippocampal signaling and the activation of either the amygdala or the BNST, depending on the total allostatic load accumulated through the pre- and postnatal life history of the animal, could determine whether the animal would show PPI or basal startle changes or unaffected ASR. A relatively low allostatic load could be supposed to activate the amygdala without activating the BNST, resulting solely in PPI changes. Increasing the allostatic load could tip the balance towards activation of the BNST and increased likelihood of changes in basal startle (Fig. 11). Accordingly, PPI changes in female blood sampled CON animals or non-sampled female and male PNM animals, exposed to pure CMS, could be due to primary activation of the amygdala as the animals had only accumulated a low allostatic load (either a low pre- or a single postnatal stressful experience) (papers I and IV). The alteration of PPI in blood sampled PNM animals exposed to pure CMS could result from an interplay between signaling from the hippocampus and the amygdala (paper I),

whereas the increased basal startle in blood sampled PNM animals, exposed to CMS and saline injections or DEX during gestation, could be effected by activation of the BNST due to the higher allostatic load accumulated in the animals combined with hippocampal CRH signaling (due to the contextual similarities between blood sampling and ASR) (Hougaard et al. 2005a,b and paper II).

Increased basal startle due to BNST activation in PNM female animals seems, however, to require more than a high allostatic load to be shown, as the PNM females in paper III did not show increased basal startle irrespective of prenatal DEX dose or postnatal exposure to the FST. This hypothesis is, however, speculative and will require substantiation in further studies.

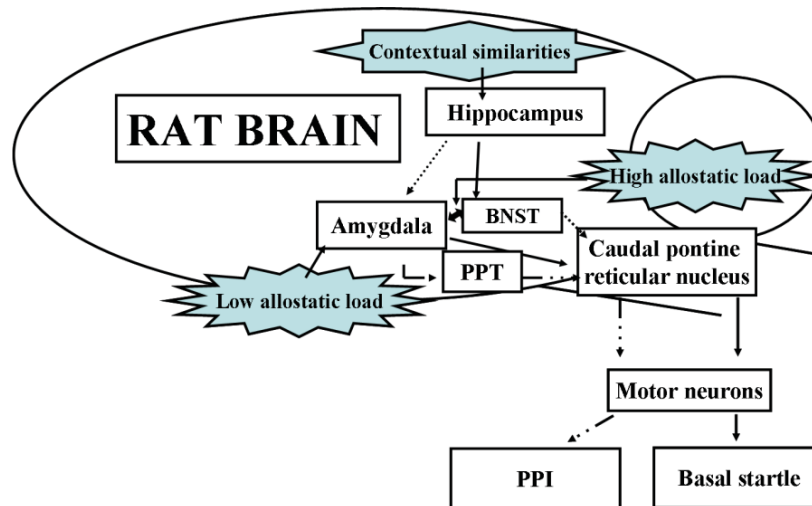


Figure 11: Schematic and highly simplified illustration of the threshold theory for modulation of either PPI or basal startle depending on allostatic load and/ or contextual clues.

### 8. 7 *The cost of an adverse life history (papers I-IV)*

Bearing the influence of postnatal experiences on the prenatal setup in mind, our model mimics the developmental consequences of some adverse life history influences. Our studies have so far shown altered ASR and increased immobility in the FST (Hougaard et al 2005a,b, papers I- IV). Yet the lack of ASR alterations in paper III, despite signs of reduced stress coping in the FST, suggests that the consequences of an adverse life history are highly complex. Central allostatic accommodation and allostatic load, e.g. the cost of adapting to change, are not pathological mechanisms in themselves but may

lead to vulnerability to pathology (Ganzel et al. 2010), for instance by affecting epigenetic changes that might be less adaptive in the long term. According to Meaney et al (2007), development occurs within an environmental context that ultimately shapes the function of the brain and other organs in the juvenile and adult animal, which ultimately defines individual differences in multiple traits, and thus vulnerability for illness over the lifespan.

The collective data from my studies indicate that the combined load of prenatal and postnatal manipulations lead to an increased propensity for altered ASR, especially if the postnatal aversive experiences bear some sort of similarity to each other.

## 9. Conclusions

Four papers described our investigations of the influence of pre- and postnatal manipulations on the acoustic startle response in adult rats.

Papers	Aim	Conclusion
I	Investigated whether chronic hyperactivity in the HPA axis, inferred from elevated CORT levels, as a result of the exposure to blood sampling under restraint, could be hypothesized to alter the ASR of female PNM rats.	Blood sampling under restraint did not induce long-term hyperactivity in the HPA-axis in PNM rats, thus changes in ASR was not associated with increased CORT levels.
II	Investigated whether observation of increased basal startle in PNM female animals depended on the phase of testing during the diurnal cycle, and whether hippocampal GR and MR expression were associated with increased basal startle.	ASR testing during diurnal light phase did not seem to be the sole requirement for observation of increased basal startle in PNM animals and hippocampal GR and MR expression were not associated with increased basal startle but CRHBP might play an important role.
III	Investigated if prior exposure to the FST, a postnatal stressor without contextual similarities with the ASR test, would induce changes in the ASR in PNM female animals.	Prior exposure to FST, lacking contextual similarities with the ASR test, did not induce changes in ASR in PNM animals in spite of reduced mobility in the FST.

IV	Investigated whether male PNM offspring would present with similar ASR changes as observed in female siblings from paper I.	No gender difference was found on plasma CORT levels. PPI alterations, similar to those observed in PNM females in paper I, were observed in PNM males.
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Altogether, the studies in this thesis have shown in an animal model that PNM can result in long term alteration of ASR in the form of PPI changes or increased basal startle. In addition to the PNM, the expression of ASR alterations can be influenced by postnatal experiences.

The recurring message in this work is that the interplay between prenatal and postnatal environmental and genetic factors is extremely complex. Allostatic load accumulated during the life history of the animals, contextual similarity between the postnatal manipulations or a combination of both seems to determine whether the behavioral result in the adult PNM animals is decreased PPI, increased basal startle or no ASR alterations compared with CON animals.

## 10. Perspectives and future studies

Our studies have shown an increased propensity for altered ASR in adult PNM animals. Exaggerated startle, particularly in relation to contextual threats, has been proposed to represent an endophenotype for anxiety disorders (Davis et al. 2010). Decreased PPI have been associated with the presence of anxiety disorders (Grillon et al. 1997;Grillon et al. 1998;Swerdlow et al. 2001;Ludewig et al. 2002;Franklin et al. 2009) and increased immobility in the FST has been proposed to mimic a symptom of depression. On a global scale, neuropsychiatric conditions are among the most important causes of disability, accounting for around one third of years lost due to disability among adults aged 15 years and over (World Health Organization 2008). Highly prevalent among these diseases are anxiety and depression (World Health Organization 2008) which are both precipitated and exacerbated by stress (Allredge 2010). Increased knowledge about this area is clearly very important. Our model might contribute with valuable input to the field of models for symptoms of anxiety disorders.

The repeated observations of an association between blood sampling under restraint and increased basal startle in PNM animals suggest that contextual clues in adverse surroundings might have a stronger impact on PNM animals than CON. It would therefore be relevant to test whether contextual fear is more strongly expressed in PNM animals.

Increased startle seems a heritable marker for anxiety in humans (Grillon et al. 1997;Grillon et al. 1998). Overall startle levels were elevated in adolescent girls and fear potentiated startle in adolescent boys of parents with anxiety disorders (Grillon et al. 1998). In animal studies, prenatal exposure to DEX has been shown to influence the second generation in the form of reduced birth weight and impaired glucose tolerance (Pollard 1986). It would be highly interesting to examine whether the phenotype of altered ASR, in response to some adverse life history influences as mimicked by our model, is an expression of genomic alterations with possible impact on the second generation.

## Summary in English

*Introduction:* Prenatal stress is a possible candidate for a predisposing condition for mental health effects later in life. PNM has been shown to decrease adaptation to new situations, and to increase emotionality in humans as well as in animals.

*Background:* Prior to the start of this PhD, two studies observed an association between prenatal manipulation (PNM) (stress or dexamethasone (DEX)), postnatal blood sampling under restraint (BS) and increased basal startle in adult rats. The startle reflex is highly sensitive to fear and anxiety in humans and animals.

*Aims:* To further investigate the animal model of combined pre- and postnatal manipulations developed in the laboratory at the National Research Centre of the Working Environment. The results have been described in 4 papers (papers I- IV). In I) it was investigated whether chronic hyperactivity in the hypothalamic pituitary adrenal (HPA) axis, inferred from elevated plasma CORT levels, as a result of the exposure to BS, could be hypothesized to alter the acoustic startle response (ASR) of female PNM rats. In II) it was investigated whether observation of increased basal startle in PNM female animals depended on the phase of testing during the diurnal cycle, and whether hippocampal glucocorticoid receptor (GR) and mineralcorticoid receptor (MR) expressions were associated with increased basal startle. In III) it was investigated if prior exposure to the forced swim test (FST), a postnatal stressor without contextual similarities with the ASR test, would induce changes in ASR in PNM female animals and in IV) it was investigated whether male PNM offspring would show similar ASR changes as observed in female siblings from paper I.

*Methods:* Rats were exposed to PNM in the form of either chronic mild stress or Dexamethasone. The adult offspring were tested for ASR after prior exposure to BS (I, II and IV) or FST (III).

*Results:* I) Changes in ASR was not associated with increased CORT levels. II) ASR testing during the diurnal light phase did not seem to be the sole requirement for

observation of increased basal startle in PNM animals. III) Prior exposure to FST, lacking contextual similarities with the ASR test, did not induce changes in ASR in PNM animals in spite of reduced mobility in the FST. IV) No gender difference was found on plasma CORT levels. PPI alterations, similar to those observed in PNM females in I), were observed in PNM males.

*Conclusions:* Our combined findings suggest that the observation of altered ASR in PNM offspring is influenced by the allostatic load accumulated during the life history of the animals, contextual similarity between the postnatal manipulations or a combination of both. The interplay between these determines if the behavioral phenotype is decreased PPI, increased basal startle or no ASR alterations compared with CON.

## Resume

*Introduktion:* Prænatal stress kan være en prædisponerende faktor for at udvikle mentale helbredsproblemer senere i livet. Studier i både mennesker og dyr har vist at prænatal stress mindsker evnen til at tilpasse sig nye situationer og øger følelsesmæssige reaktioner.

*Baggrund:* Inden starten af denne phd, fandt man i to studier en forbindelse mellem prænatal manipulering (PNM) (i form af stress eller dexamethason), blodprøve under fastspænding (BS) og øget basal startle i voksne rotter. Startle refleksen er meget følsom overfor angst og frygt i mennesker og dyr.

*Formål:* Formålet var at arbejde videre med denne dyremodel som kombinerede præ- og postnatale påvirkninger. Resultaterne fra mine studier er blevet beskrevet i 4 artikler (artikel I- IV). I) Her blev det undersøgt om kronisk hyperaktivitet i HPA akse (hypothalamus-hypofyse-binyrebark akse), set i forhold til øget plasma kortikosteron niveau ved blodprøvetagning under fastspænding, kunne formodes at ændre PNM hunrotters akustiske startle respons (ASR). II) Her blev det undersøgt om observationen af øget basal startle i PNM hunrotter afhang af den fase af døgnnet hvor dyrene blev testet. Det blev også undersøgt om udtrykket af glukokortikoid og mineralkortikoid receptorerne i hippocampus var associeret med øget basal startle. III) Her blev det undersøgt om det at have været testet i ”forced swim” testen (FST), en postnatal stressoplevelse uden lighed med ASR testen, ville føre til ændringer i ASR i PNM hunrotter. IV) Her blev det undersøgt om PNM hanrotter ville udvise samme ASR ændringer som der blev observeret i artikel I i hannernes kuldsøstre.

*Metode:* Rotter blev udsat for PNM i form af enten kronisk mild stress eller Dexamethason. De voksne afkom blev testet for ASR efter tidligere at have været udsat for enten BS (I, II og IV) eller FST (III).

*Resultater:* I) Ændringer i ASR var ikke associeret med øget kortikosteron niveau. II) Hvilken fase af døgnnet ASR testen blev udført under så ikke ud til at have afgørende indflydelse på observationen af øget basal startle i PNM hunrotter. III) At have været

## Summary (Dk)

udsat for FST, der ikke minder om ASR testen, inden ASR testen førte ikke til ASR ændringer i PNM hunrotter, på trods af mindsket mobilitet i FST i de samme dyr. IV) Der blev ikke observeret nogen forskel på kønnene med hensyn plasma kortikosteron niveau. PNM hanrotter udviste PPI ændringer i samme stil som deres kuldsøstre i artikel I).

*Konklusion:* Vores fund tyder på at den samlede stress påvirkning dyret har været udsat for gennem hele sin livshistorie, ligheder mellem postnatale oplevelser eller kombinationen af begge faktorer har indflydelse på hvilke ASR ændringer der viser sig i PNM dyr. Samspillet imellem disse har indflydelse på om dyrenes adfærdsmæssige phenotype er mindsket PPI, øget basal startle eller ingen ASR ændringer i forhold til kontrol dyr.

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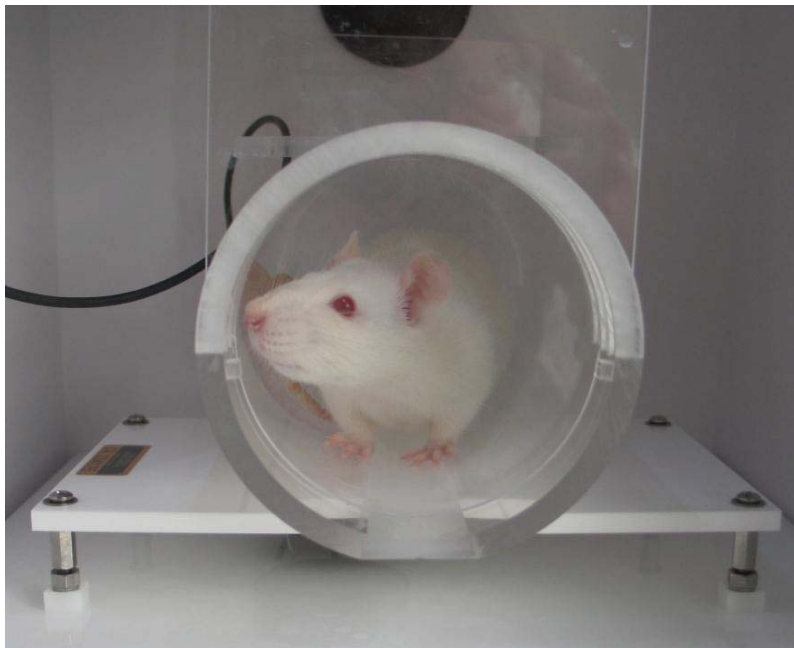
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## Appendix

On the following pages the papers I – IV are presented.

- Paper I) Kjær SL, Wegener G, Rosenberg R, Lund SP, Hougaard KS (2010)  
**Prenatal and Adult stress interplay - Behavioral implications**  
*Brain Research 1320:106-13*
- Paper II) Kjær SL, Hougaard KS, Tasker RA, MacDonald D, Rosenberg R, Elfving B, Wegener G (2011)  
**Influence of diurnal phase on startle response in adult rats exposed to dexamethasone in utero**  
*Physiology & Behavior 102(5): 444-452*
- Paper III) Kjær SL, Wegener G, Rosenberg R, Hougaard KS (2010)  
**Reduced Mobility But Unaffected Startle Response in Female Rats Exposed to Prenatal Dexamethasone: Different Sides to a Phenotype**  
*Developmental Neuroscience 32:208-216*
- Paper IV) Hougaard KS, Mandrup KR, Kjaer SL, Bøgh IB, Rosenberg R, Wegener G (2010)  
**Gestational chronic mild stress: Effects on acoustic startle in male offspring of rats.**  
*International Journal of Developmental Neuroscience, in press,*  
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**Research Report**
**Prenatal and adult stress interplay — behavioral implications**
**S.L. Kjøræ<sup>a,b</sup>, G. Wegener<sup>a</sup>, R. Rosenberg<sup>a</sup>, S.P. Lund<sup>b</sup>, K.S. Hougaard<sup>b,\*</sup>**
<sup>a</sup>Centre for Psychiatric Research, Aarhus University Hospital Risskov, Skovagervej 2, 8240 Risskov, Denmark

<sup>b</sup>National Research Centre for the Working Environment, Lersø Parkallé 105, 2100 Cph, Denmark

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**ABSTRACT**

The origin of adult behavior and the possible pathogenesis of psychiatric disorders remain elusive, but extensive research indicates that interaction of genes and environment play a crucial role for adult phenotype. Differences in susceptibility may arise by earlier experiences and genomic variables, either alone or in combination. The acoustic startle response (ASR) has been shown to be altered in patients with several psychiatric diseases, a change that could result from a persistent sensitization caused by chronic arousal secondary to a traumatic incident. The current work hypothesized that a single aversive procedure would induce long-term hyperactivity in the HPA-axis of rats that had become vulnerable by prenatal stress, and thereby change reactivity in the ASR. Prenatal stress was achieved by maternal gestational exposure to Chronic Mild Stress (CMS). At age 3 months, the offspring were blood sampled by a stressful procedure, and subsequently tested in the acoustic startle paradigm. Prenatal CMS strongly reduced prepulse inhibition (PPI) whereas postnatal blood sampling under restraint generally increased PPI. Our data demonstrate interplay between pre- and postnatal stressful events, but also that this interaction is complex and could influence the interplay between PPI and basal startle. Our results suggest that circumstances dating back to early development may have implications for adult life behavior, and based on this we propose a new theory of a threshold in the induction of a stress response in the ASR test, which influences whether the PPI or basal startle response will be affected.

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**1. Introduction**

The importance of the uterine environment for development of mental and physical diseases later in life is supported by evidence from an increasing number of studies (O'Connor et al., 2002; Talge et al., 2007; Van den Bergh et al., 2005). Epidemiological studies have shown that low birth weight is linked with subsequent adult disorders, including hypertension and type 2 diabetes (Barker, 1995; Hales et al., 1991). Low weight during year one was associated with adult suicide and depression (Barker et al., 1995). These links suggest that

factors acting early in life “program” the set-point of physiological systems, perhaps to prepare the individual for life under sub-optimal conditions.

Environmental interactions with the genome are essential in development of pathophysiology. It implicates that a genetic vulnerability or other predisposition may interact with the environment and stressful life events to trigger mental disease. This hypothesis include the “two hit” disease model, originally proposed in order to model the possible multifactorial origin of schizophrenia (Bayer et al., 1999; Maynard et al., 2001). Recently, however, the model was also

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\* Corresponding author. Fax: +45 39 16 52 01.

E-mail address: [ksh@nrcwe.dk](mailto:ksh@nrcwe.dk) (K.S. Hougaard).

used in a broader scope to include development of stress related psychopathology, e.g. depression and anxiety (Cirulli et al., 2009).

Prenatal stress (PNS) is a possible candidate for a predisposing condition for mental health effects later in life. PNS has been shown to increase the hypothalamic pituitary-adrenal (HPA) axis response in several studies, often manifested as enhanced or prolonged corticosterone response to acute stressors (Henry et al., 1994; Koenig et al., 2005), to decrease adaptation to new situations, and to increase emotionality in animals (Burton et al., 2006; Cabrera et al., 1999; Damaudery and Maccari, 2008; Lehmann et al., 2000; Maccari et al., 2003; Mastorci et al., 2009) as well as humans (Meijer, 1985; Van den Bergh et al., 2005; Weinstock, 2001). We have previously observed increases in the acoustic startle response (ASR) in prenatally stressed rats, but only when the response was tested after a postnatal stressful event (Hougaard et al., 2005a, 2005b). The acoustic startle response is a characteristic sequential contraction of the skeletal musculature evoked by a sudden and intense acoustic stimulus (Koch, 1999). It appears in a wide range of species, and exaggerated startle is the result of inappropriate activation of a normally adaptive defense system. The startle reflex is highly sensitive to fear and anxiety in both humans and animals. Elevated startle magnitude has been proposed to be a marker for anxiety disorders in humans, and possibly for major depressive disorder (Grillon, 2002; Grillon et al., 2005).

The startle reaction may be inhibited by a prepulse-stimulus (pre-pulse inhibition (PPI)) which reflects an operational measure of the sensorimotor gating system (Braff et al., 1992). PPI has been reported to be associated with anxiety in humans (Franklin et al., 2009; Ludewig et al., 2002). Altogether, these findings conclude that the startle reaction is a sensitive marker of sensory motor changes in the central nervous system in both humans and rats (Koch, 1999).

Based on the hypothesis of Grillon (2002) that elevated startle in anxious patients may result from a persistent sensitization caused by chronic arousal secondary to a traumatic incident, the present study was initiated to investigate whether the procedure of blood sampling under restraint would induce long-term hyperactivity in the HPA-axis and change the acoustic startle reactivity of prenatally stressed rats. Based on earlier findings (Hougaard et al., 2005a, 2005b) we expected blood sampling to be associated with increased basal startle in PNS offspring and subsequent changes in corticosterone (CORT) response revealed during a second blood sampling in the same group of animals.

## 2. Results

### 2.1. Corticosterone

CORT was measured in plasma from blood sampling under restraint at 3 and 8 months of age, c.f. Table 1. The concentration of plasma CORT differed between samples, as expected at both ages (3 months:  $[F(2,54)=141.891; P<0.001]$ ; 8 months:  $[F(2,94)=83.66; P<0.001]$ , Figs. 1A and B, respectively). No statistically significant difference was related to prenatal CMS at either time point.

### 2.2. Acoustic startle response

#### 2.2.1. Basal startle

The rats were tested at the age of 6 months, during the active (dark) phase. Half of the offspring had been blood sampled 3 months previously, c.f. Table 1. The average basal startle response during the middle 10 startle trials was similar in all groups (Fig. 2). Comparable outcomes were registered for the 5 initial and the concluding 5 startle trials as well as during the second startle session 1 week later.

#### 2.2.2. Prepulse inhibition

Prepulse inhibition of the startle reaction to a 120 dB noise pulse was investigated for four levels of prepulses (72, 74, 78, and 86 dB) (Fig. 3).

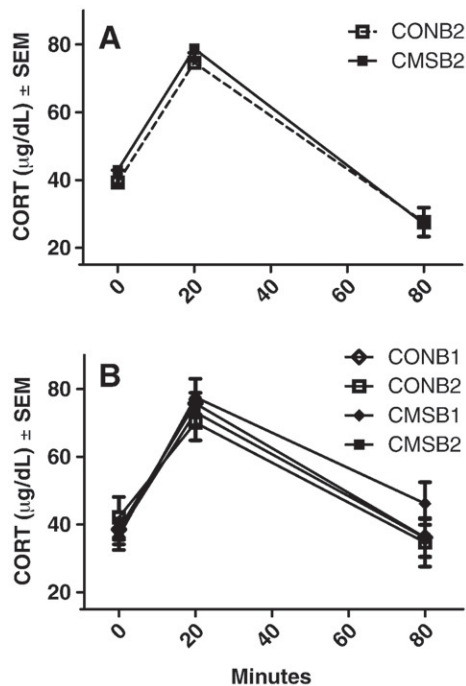
Visual inspection of Fig. 3 indicates that the prepulse of 72 dB is associated with negative values of PPI in CMS offspring, but not in controls, suggesting that this low prepulse increased the response to the 120 dB startle pulse in the prenatally stressed offspring (Fig. 3A). The louder prepulses inhibited startle response overall, however less so in CMS than in control offspring (Figs. 3B–D). We found statistically significant differences for PPI72, PPI78, and PPI86 (CMS:  $[F(1,59)=6.774; P=0.012]$ ,  $[F(1,59)=8.585; P=0.005]$ , and  $[F(1,59)=5.997; P=0.017]$ , respectively). Pairwise comparisons regarding effect of prenatal CMS within blood-sampled or non-sampled animals showed specific effects of CMS in the form of less inhibition between CONB2 and CMSB2 for PPI72 ( $P=0.048$ ) and PPI78 ( $P=0.018$ ).

Blood sampling 3 months previously increased PPI at the two highest prepulse intensities irrespective of prenatal exposure, c.f. Figs. 3C and D (blood sampling:  $[F(1,59)=8.179; P=0.006]$  and  $[F(1,59)=3.802; P=0.056]$  for PPI78 and PPI86, respectively). Pairwise comparisons regarding the effect of

**Table 1 – Group exposures and timeline.**

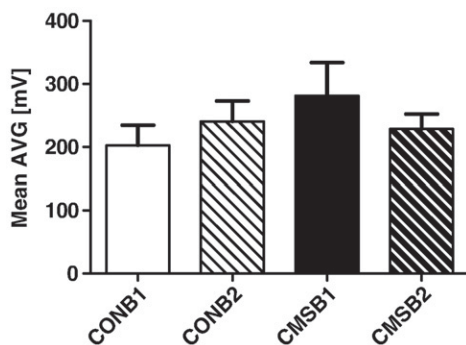
	Fertilization	CMS	Birth	BS	ASR	BS
	GD 0	GD 9–21	0	3 months	6 months	8 months
CONB1					X	X
CONB2				X	X	X
CMSB1		X			X	X
CMSB2		X		X	X	X

CON: Control, CMS: Chronic Mild Stress, B1: Blood sampled once, B2: Blood sampled twice. GD: gestational day. BS: Blood sampling. ASR: Acoustic startle response.



**Fig. 1 – Mean corticosterone response to blood sampling under restraint in female rat offspring at age 3 months (A) and 8 months (B).** CONB1=control+ blood sampled at 8 months. CONB2=control+ blood sampled at both 3 and 8 months. CMSB1= prenatal stress+ blood sampled at 8 months. CMSB2= prenatal stress+ blood sampled at both 3 and 8 months. T0=0 min, basal sample; T20=after 20 min restraint, stress sample; T80=60 min after the restraint, post stress sample. Mean  $\pm$  SEM,  $n=16$ .

blood sampling within each prenatal exposure group showed that PPI was increased in blood sampled compared to non-sampled control offspring at PP78 (CONB1 vs. CONB2:  $P=0.017$ ). The second week of testing revealed no systematic differences between groups (data not shown).



**Fig. 2 – Mean acoustic startle response to the middle 10 pure 120 dB impulses in female rats during the first week of testing (active (dark) phase) at age 6 months.** CONB1=control, CONB2=con+ blood sampled at 3 months. CMS=prenatally stressed (PNS) with chronic mild stress. CMSB1= prenatal stress. CMSB2= prenatal stress+ blood sampled at 3 months. Bars=mean  $\pm$  SEM,  $n=16$ .

### 2.3. Vaginal impedance

The animals were examined for vaginal impedance directly after each startle session. There were an equal number of animals in proestrous and not in proestrous in each group during week 1. In week 2, two thirds of the animals were in proestrous. We found no effect of estrous stage on startle or PPI when impedance was included as a cofactor (data not shown).

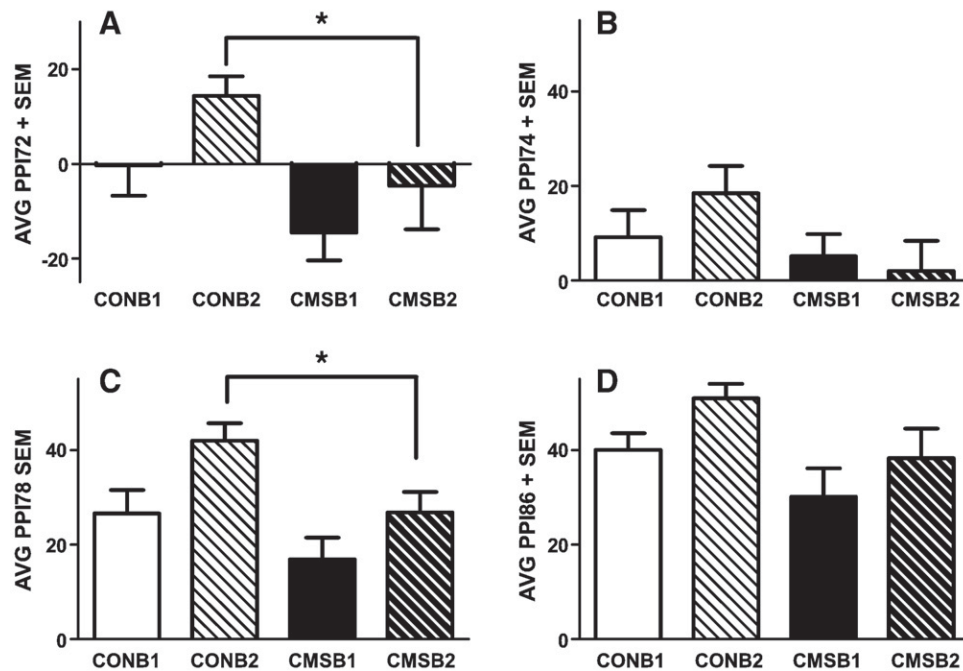
## 3. Discussion

The main finding of the present study is reduced PPI in rats prenatally stressed by CMS. PPI reduction was noted at three levels of prepulses (PPI74, PPI78 and PPI86), although basal startle reactivity was unaffected by prenatal exposure.

The combination of prenatal CMS with postnatal blood sampling under restraint has been observed to increase basal startle reaction in two earlier studies (Hougaard et al., 2005a, 2005b). Previous work have demonstrated that the reactivity of the HPA axis may be increased in prenatally stressed rats (Henry et al., 1994; Koenig et al., 2005) in parallel with an increased responsiveness of the CRHergic system (summarized in (Hougaard et al., 2005b)). Administration of corticotrophin releasing hormone (CRH) has been shown to intensify the acoustic startle response in rats (Dunn and Berridge, 1990; Lee and Davis, 1997; Servatius et al., 2005). In the present study we therefore wanted to examine whether the stressful procedure of blood sampling under restraint would induce long-term hyperactivity in the HPA-axis of prenatally stressed rats, which in turn would affect the acoustic startle reactivity of the test subjects. However, the CORT response showed resilience to blood sampling, indicating that the reactivity of the HPA axis in the animals was not permanently altered by the pre- and postnatal stressful exposures despite altered PPI in prenatally stressed offspring. These results are in full agreement with our previous studies, where PNS and CON rats showed similar CORT levels prior to ASR testing (Hougaard et al., 2005a, 2005b). Our results therefore seems to imply that long-term consequences of prenatal stress on the startle response do not require set-point changes in the HPA axis (Rimondini et al., 2003). In fact Lee et al. (1994) have shown that ASR changes may occur independently of the integrity of the HPA axis, because CRH-enhanced startle in rats was observed even in absence of adrenal glands.

Prenatal CMS was associated with reduced PPI, which differed from our earlier findings of increased basal startle reactivity (Hougaard et al., 2005b). The exact association between PPI and startle has yet to be elucidated. The PPI phenomenon depends on (i) the intensity of the startle pulse and prepulse, (ii) the time interval in between, and (iii) the intensity of the background noise. Numerous animal studies using behavioral endpoints to investigate effects of various prenatal stressors, including pharmacological stress, have reported divergent results of either basal startle or PPI, indicating a complex connection between the applied stressors and induction of an increased startle response (Hauser et al., 2006; Hougaard et al., 2005a; 2005b; Koenig et al., 2005; Lehmann et al., 2000).

From a behavioral viewpoint, startle represents a protective response (Koch and Schnitzler, 1997), being reflectory and



**Fig. 3** – Mean average PPI72 A), PPI74 B), PPI78 C) and PPI86 D) in female offspring at the first week of testing during the dark phase at age 6 months. CONB1=control, CONB2=control+ blood sampled at 3 months. AVG=average. PPI=prepulse inhibition. CMS=prenatally stressed with chronic mild stress. CMSB1=prenatal stress. CMSB2=prenatal stress+ blood sampled at 3 months. General effect of prenatal background on PPI72, PPI78, and PPI86: CMS: [ $F(1,59)=6.774$ ;  $P=0.012$ ], [ $F(1,59)=8.585$ ;  $P=0.005$ ], and [ $F(1,59)=5.997$ ;  $P=0.017$ ], respectively. PPI78 and PPI86 also showed effect of postnatal blood sampling: [ $F(1,59)=8.179$ ;  $P=0.006$ ] and [ $F(1,59)=3.802$ ;  $P=0.056$ ], respectively. Mean  $\pm$  SEM,  $n=16$ . \* $P<0.05$ .

mediated by short neuronal pathways with few synaptic relays organized in the hindbrain (Fendt et al., 2001; Koch and Schnitzler, 1997). However, unnecessary startle may represent unnecessary use of energy reserves, and complex processing of stimuli occurs at the forebrain level to enable the most appropriate response in a given situation. PPI has been hypothesized to attenuate startle by an inhibitory effect on the startle pathway (the giant neurons in the caudal pontine reticular nucleus (PNC)), allowing time for perceptual processing after the stimulus (Fendt et al., 2001; Graham, 1975; Koch and Schnitzler, 1997). Increased PPI could therefore be a sign of stronger inhibition in the PNC, but the rationale for a decreased PPI is evidently far less obvious. It has been suggested that if the prepulse is recognized as novel during processing in the forebrain, inhibition of the PPI would occur, which subsequently would allow the animal to startle to protect itself (Larrauri and Schmajuk, 2006). PPI may therefore represent a mechanism that allows the animal to process information before reacting, and under appropriate circumstances, the inhibitory effect of the prepulse may be reduced (Larrauri and Schmajuk, 2006).

Although basal startle and PPI may have to be viewed as two separate behavioral functions, there seems to be a strong interplay between them, an interplay which may have great influence on the overall behavioral profile of the animal. We have not been able to locate studies on the effects of various forms of prenatal stress in rats, including pharmacological stress, that have shown simultaneous changes in basal startle and PPI (Hauser et al., 2006; Hougaard et al., 2005a, 2005b; Koenig

et al., 2005; Lehmann et al., 2000). However, concomitantly enhanced startle and reduced PPI has been observed in male rats after 8 weeks of isolation rearing starting at weaning (Du et al., 2010). The learning-induced enhancement of PPI was not present in this study, even though basal startle was enhanced, which led to the conclusion that the neural network for top-down modulation of PPI might differ from the neural network underlying the startle responses. Furthermore, basal startle may be top-down regulated by more than one neural network. The latter is pointed out by Lee and Davis (1997), who investigated two different forms of startle enhancement: fear potentiated and CRH-enhanced startle. Lesions of the bed nucleus of the stria terminalis (BNST) completely blocked CRH-enhanced startle, but not lesions in the central amygdala (CeA) or basolateral amygdala (BLA). Conversely, similar lesions of the BNST did not block fear-potentiated startle, whereas lesions of the CeA or BLA did. This suggests that CRH-enhanced startle might be a measure of anxiety, whereas fear-potentiated startle could be a measure of fear, leaving BNST a possible neural substrate related to anxiety states, whereas CeA/BLA could be critical for fear responses. BNST has later been hypothesized to mediate slower-onset, longer-lasting responses, which frequently accompany sustained threats and persist even after threat termination (Walker et al., 2003). It could therefore also be hypothesized that the BNST might be a possible candidate for mediating long-lasting effects of prenatal stress observed in our studies, explaining why enhanced startle observed 3 months after the blood sampling may have occurred without alterations in CORT (Hougaard et al., 2005a, 2005b).

However, none of the cited studies have dealt with effects of prenatal stress, and can therefore and may therefore not account for the prenatal effects on PPI. The amygdala has been implicated in modulation of PPI (Du et al., 2010; Li et al., 2009), as well as fear enhanced startle (Lee and Davis, 1997), but this does not explain why some studies observe changes in either basal startle or PPI whereas others find changes in both. The CeA and BNST are both described as glucocorticoid-sensitive (Lechner and Valentino, 1999) with BNST suggested to be activated following amygdaloid CRH release (Lee and Davis (1997). Glucocorticoid receptors (GR) have been suggested to be capable of eliciting tissue-selective effects as the GR promoter shows multiple tissue specific alternate untranslated first exons in rats (McCormick et al., 2000). These transcripts all give rise to the same receptor protein, as only exons 2–9 encode the full protein (O'Regan et al., 2001). Given the stimulation pattern above, it may be hypothesized that specific GR receptors could be activated in the amygdala when specific sensory cues are present, and further that the varying effects of prenatal stress on PPI and startle may be explained by the existence of a certain threshold in the induction of a stress response on the GR receptors' expression in the amygdala and BNST. However, this hypothesis is speculative and will require substantiation in further studies.

Compared with previous studies (Hougaard et al., 2005a, 2005b) two major methodological differences exist. Firstly, in the present study stressed dams solely underwent CMS, whereas control dams stayed undisturbed in their cages. This contrasts the treatment in our previous studies, where both stressed and control dams were additionally stressed by daily allocation to wire cages in a different animal room (Hougaard et al., 2005a) or by s.c. injections with saline (Hougaard et al., 2005b). The change in response following the change in treatment suggests that pure CMS is indeed stressful and induces a change in PPI, but maybe less stressful than adding other components to the CMS-regimen, as illustrated by our earlier observations.

Secondly, ASR testing was performed during the dark phase whereas ASR testing previously was conducted during the light phase (Hougaard et al., 2005a, 2005b). Testing during the light phase may increase basal startle in prenatally stressed animals compared to controls, because rats are nocturnal animals and light seems aversive to them, hence the phenomenon of light enhanced startle (Tazumi et al., 2005; Walker and Davis, 1997). Testing during the resting period of the rats (light) may have constituted a challenge in itself, which could influence PNS animals more than their CON counterparts. Other studies which have tested prenatally stressed rats during the dark phase have also reported changes in PPI but not in basal startle (Hauser et al., 2006; Lehmann et al., 2000).

In the present study, blood sampling under restraint increased PPI in both PNS and CON and thus apparently affected PPI in the opposite direction of CMS. A somewhat parallel finding of pre- and postnatal interaction in the form of increased PPI in offspring from gestationally restrained dams, with amelioration hereof by maternal separation have previously been reported by Lehmann and co-workers (2000). Adverse effects of stress have lately received increased attention, leaving the positive and facilitatory effects of stress largely unnoticed. A positive effect of stress on arousal has been proposed by Hans Selye in 1936 (Selye, 1936). Later studies clearly state that

corticosteroids also protect the brain against adverse events, and are essential for adaptive cognitive performance (De Kloet et al., 1999; McEwen et al., 1992; McEwen, 2008). Indeed, prenatal stress has in a previous study been associated with enhanced cognitive function in the Morris water maze (Fujioka et al., 2001; Hougaard et al., 2005a) which contrasts the more hazardous effects described above, such as decreased adaptation to new situations and increased emotionality. On the other hand, even though blood sampling overall increased PPI in the offspring, the PPI level is still lower in PNS offspring than in blood sampled CON offspring. We do not have a consistent explanation for this, but the efficacy of prenatal manipulation to decrease PPI in the face of the blood sampling could be interpreted as a possible stronger relative impact of the prenatal background than the postnatal experience or that the postnatal stressor affects the PPI by a different neurological pathway than the PNS exposure. Thus, the effects of stress may not solely depend on dose but also on timing and neurological target.

The second cycle of ASR testing did not yield statistically significant changes in PPI even though PPI could be conceived as a stressful procedure in line with other postnatal stressors like blood sampling. Again, we cannot suggest a consistent explanation to this observation, but the results are in line with our previous study (Hougaard et al., 2005b), where effects of blood sampling also ceased to be statistically significant when it was performed in the second week post parture.

This study was conducted in female rats in line with our previous studies (Hougaard et al., 2005a, 2005b). Female rats were chosen as epidemiological data have shown that women have a higher overall prevalence rates for anxiety disorders than men (Pigott, 2003). Ovarian hormones might influence PPI, but the role of estrous phase in PPI is under debate. Basal startle and PPI showed, in the present study, no dependence on impedance as a proxy for female cyclicity. Kinkead et al. (2008) reported reduced PPI fluctuations across the day in proestrous compared with diestrous females with higher PPI in proestrous than diestrous females, and time of day of testing was found to be a significant factor in determining PPI differences, but 3 h after lights were switched on, no PPI difference was found between proestrous and diestrous females (Kinkead et al., 2008). Similarly Adams et al. (2008) found no effect of estrous phase on PPI when testing was carried out within a 4-h window following onset of the dark cycle (Adams et al., 2008), indicating that the effects of estrous-cycle differences in PPI is smaller, when testing is delayed from changes in the light-cycle.

Blood sampling under restraint was used as postnatal stressor in the work presented here. Restraint is aversive to rats (Glavin et al., 1994), and part of the experimental procedure of ASR measurement bear resemblance to aversive elements of the blood sampling procedure, i.e. confinement in a test tube. This could suggest that the effects on ASR are caused by aversive contextual similarities between the blood sampling under restraint and the ASR test. Future studies should consider whether a similar effect on ASR would be elicited if the blood sampling were to be substituted with another type of stressor, or if the effect is somehow a product of the sampling procedure. Restraint alone might be sufficient to cause the effect on PPI if restraint is the dominant contextual clue linking the tests.

All together the presented data demonstrate interaction between pre- and postnatal stressful events, but also that

interplay between stressors may be very complex. Small differences in stressor protocols or even control conditions may alter the resulting phenotypes as well as the context of postnatal stressful experiences. Future studies should be diverted to the interplay between pre- and postnatal stress exposures in order to elucidate the complexity of adult behavior.

## 4. Experimental procedures

### 4.1. Animals

Time-mated young adult rats (44 Wistar, HanTac:WH, SPF) arrived at gestation day (GD) 3. The rats were randomly distributed pair wise to white plastic cages (27×43×18 cm, Scanbur, Denmark) with pine-bedding (Lignocel S8, Brogaarden®, Denmark). The cages also contained nesting material (Enviro-Dri, Brogaarden®, Denmark). Environmental conditions were automatically controlled with a 12-h light–dark cycle with lights off at 06.00 am. Food (Altromin Standard Diet 1324) and tap water were provided ad libitum. Clean cages and new bedding were provided twice weekly. The animal welfare committee, appointed by the Danish Ministry of Justice, granted ethical permission for the studies. All procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Danish law regulating experiments on animals.

### 4.2. Prenatal stress exposure

#### 4.2.1. Chronic mild stress

The day after arrival (GD 4), the rats were weighed and allocated to 2 groups: 20 controls (CON) and 24 animals which were subjected to chronic mild stress (CMS) at GD 9–21. Body weights were also recorded at GD 7, 10, 13, 17, 19, and 21. The CMS model is a schedule of chronic stress, where various relatively mild stressors are presented in a random schedule and has been described in detail previously (Hougaard et al., 2005b). After termination of exposures at GD 21, the females were singly housed.

### 4.3. Prewaning data

Thirty-two of the pregnant dams (16 CON and 16 CMS) delivered litters with at least 7 pups of which 3 were females. These were subsequently used for the study. The animals were left undisturbed until weaning at 3 weeks of age except for general animal husbandry.

### 4.4. Post-weaning investigations

At weaning, one female per litter was selected at random for each postnatal group and housed in non-sibling pairs of similar prenatal exposure and postnatal testing. The animals were housed under conditions as described above, except for addition of aspen chewing bars (Brogaarden®, Denmark). The animals were divided into groups determined by prenatal exposure (CON or CMS) and number of postnatal blood samplings (B1 or B2, see Table 1).

Investigations were performed during the dark part of the diurnal cycle (between 08.00 am and 05.00 pm). Time of testing on the day was counterbalanced between experimental groups.

### 4.5. Blood sampling and analysis for blood corticosterone

At the age of 3 months, tail blood was sampled thrice from CONB2 and CMSB2 offspring, as this procedure in combination with prenatal stress has been found to increase the basal startle response in previous studies (Hougaard et al., 2005a, 2005b). Sampling took place between 08.30 am and 03.00 pm with the animals in their dark phase. The procedure was performed as previously described (Hougaard et al., 2005b). Briefly, each animal was carried from the colony room to a separate laboratory, restrained in an immobilizer (model IM/OH, Scanbur A/S, Denmark), and blood was collected from a vertical incision in one of the tail veins (basal sample). After an additional 20 min of restraint, blood was collected again (stress sample). The rat was subsequently returned to its cage, and one hour later brought back to the laboratory, restrained and sampled for the post stress sample. Sampling was performed within 2 min from the animal was picked up from its home cage. To investigate whether the first blood sampling had induced long-term hyperactivity of the HPA-axis, the blood sampling procedure was repeated at the age of 8 months for CONB2 and CMSB2 and performed for the first time for CONB1 and CMSB1, the latter two groups serving as blood sampling controls.

Corticosterone in plasma was determined by competitive radioimmunoassay performed as previously described (Pedersen et al., 2000). Commercially certified reference materials for the internal quality assurance were not available. Hence, the reference materials of two concentrations (32.2 µg/dL og 115 µg/dL corticosterone, respectively) were prepared from two rats. The samples were analyzed in duplicate. The measured concentrations were entered in a Westgaard control chart to ensure precision and trueness of the method at any time. Plasma was prepared as previously described and was diluted in PBS (pH 7.4; (Pedersen et al., 2000)).

### 4.6. ASR and PPI

The animals were tested for ASR at the age of 6 months, using SR-Lab™ SDI startle response system (SanDiego Instruments, INC. Europe). Testing was conducted as previously described (Hougaard et al., 2005a) except that the animals stayed in the animal colony room during the entire study (in our earlier studies the animals were moved to another colony building a few weeks prior to testing). The circadian rhythm of the rats was kept reversed, i.e. lights off between 06.00 am and 06.00 pm for the full experiment. Startle tubes were cleaned with 70% ethanol between animals. Each animal was tested twice to test for persistency of effect, with 1 week between sessions.

The animals were transferred to the experimental room at least 1 h before the test. Throughout the startle protocol, white background noise (70 dB(A)) was delivered continuously inside the chambers from a 3.5" tweeter (model BT2, MG electronics, NY) 14 cm above the plexiglass tube. The internal light was on during testing in the chambers. A 5-min acclimatization period commenced the test session that lasted approximately 20 min and consisted of 45 trials. The startle eliciting stimulus consisted of a 40-ms 2–10 kHz broadband noise-burst presented at 120 dB (A). The cutoff frequency of the of the noise bursts at 10 kHz was intrinsic in the equipment, seemingly caused by a low-pass filter that has been fitted in order to

protect the tweeter. The equipment worked satisfactorily, but the set-up leaves a substantial part of the 0.5- to 70-kHz hearing range in the rats (Lund and Kristiansen, 2008) without proper stimulation, and consequently, the possibility of having even clearer test results by including also the high-frequency hearing of the rats.

Each session started and ended with 5 startle trials consisting of 120 dB(A) bursts of white noise, each lasting 40 ms followed by 35 test trials that were delivered in a semi-randomized order (10 startle trials of 120 dB(A); 5 each of 4 prepulses (72, 74, 78 and 86 dB(A), respectively)+startle trials (denoted PPI72, PPI74, PPI78, and PPI86, respectively); 5 trials with no stimulus except background noise). The movement of the tube was registered for 100 ms after onset of the startle stimulus (sampling frequency 1 kHz), amplified, and the average response over 100 ms (AVG) was calculated by computer. For each level of prepulse, the AVGs were averaged and used for calculation of PPI. PPI was expressed as the percent reduction in AVG compared to the average of the 10 middle startle trials:  $\%PPI = 100 - ((AVG \text{ at prepulse} + \text{startle-trial}) / (AVG \text{ at startle trial}) \times 100\%)$ .

#### 4.7. Measurement of vaginal impedance

Estrous stage was assessed by use of a MK-10C rat vaginal impedance checker (Muromachi Kikai Co, Ltd. Japan) immediately after each startle test session. The rat was wrapped in a towel except for the tail. The probe was inserted in the vagina until electrical impedance stabilized. The animal was then returned to its cage. The probe was cleaned with 70% ethanol between animals. Impedance values of 3 kohm or above indicated the animal was in the proestrous stage (Bartos, 1977). The technique was chosen to minimize stress to the animals, as only a single test is needed to determine the estrous phase when using the impedance checker.

#### 4.8. Statistical analysis

Startle data were analyzed by two-way ANOVA, with Group (CON and CMS) and Blood sampling (+/-) as factors. When appropriate ( $P < 0.05$  in two-way ANOVA), Fishers Least Significant Difference Test was applied for pair wise comparisons. For PPI, data were analyzed separately for each level of prepulse. Impedance was included in the analyses as cofactor. Plasma corticosterone at age 8 months was analyzed by three-way ANOVA with Group (CON and CMS) and Blood sampling (+/-) as between-subject factors, and Sample (basal, stress, and post-stress) as within-subject factor (repeated measure). At age 3 months, CORT data were analyzed with Group as factor and Sample as repeated measure.

The accepted level of statistical significance was  $<0.05$  (SYSTAT Software Package version 12).

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## Influence of diurnal phase on startle response in adult rats exposed to dexamethasone in utero

S.L. Kjær<sup>a,b</sup>, K.S. Hougaard<sup>b</sup>, R.A. Tasker<sup>c</sup>, D.S. MacDonald<sup>c</sup>, R. Rosenberg<sup>a</sup>, B. Elfving<sup>a</sup>, G. Wegener<sup>a,\*</sup>

<sup>a</sup> Centre for Psychiatric Research, Aarhus University Hospital, Risskov, Skovagervej 2, 8240 Risskov, Denmark

<sup>b</sup> National Research Centre for the Working Environment, Lersø Parkallé 105, 2100 Copenhagen, Denmark

<sup>c</sup> Department of Biomedical Sciences, University of Prince Edward Island, Charlottetown, PE, C1A 4P3 Canada

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### ABSTRACT

Depression and pathological anxiety disorders are among the most prevalent neurological diseases in the world and can be precipitated and exacerbated by stress. Prenatal stress alters both behavioral and endocrine responses to stressful stimuli in later life. We have previously observed increased basal acoustic startle response (ASR) in Wistar rats exposed to stress or dexamethasone (DEX) in utero when tested during the light phase of the circadian rhythm, and decreased prepulse inhibition (PPI) in similar animals tested during the dark phase of the cycle. We speculated that this observation of increased basal startle might be influenced by diurnal phase. In the present study, adult female Sprague Dawley rats, stressed prenatally with DEX (200 µg/kg, gestational days 14–21) and postnatally by blood sampling under restraint, were tested for the ASR during both circadian phases (light and dark). Basal startle was increased in animals tested both during the light and the dark phases of the cycle. We hereby replicated our earlier findings in a new strain and laboratory, thus strengthening the validity of our model regarding prenatal stress effects on ASR in female offspring. Our results indicate that observation of increased basal ASR is not solely dependent on diurnal phase. We found no difference in hippocampal glucocorticoid and mineralocorticoid receptor expression between groups.

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### 1. Introduction

Neuropsychiatric conditions are the most important causes of disability worldwide, accounting for approximately one third of years lost due to disability among adults [1]. With high prevalent are depression and pathological anxiety disorders [1] which are both precipitated and exacerbated by stress [2]. Inter-individual differences in coping with stress (e.g. in the occupational setting) depend on the secretion and action of stress hormones, which are largely shaped by gene–environment interactions throughout life [3]. Animal studies show that prenatal exposure to stress or dexamethasone (DEX) alters both the behavioral and endocrine responses to stressful stimuli in later life in a manner that is generally, but not always [4,5], consistent with the idea of heightened stress response and emotional behavior. Further, both prenatal stress and prenatal glucocorticoid exposure result in alterations in brain glucocorticoid receptor (GR) expression, though the observed changes within specific brain areas differ with the period of exposure [6–8].

In a series of previous studies in our laboratory, pregnant rats were exposed to either chronic mild stress (CMS) or DEX. Their female

offspring presented with behavioral changes in the form of increased basal acoustic startle response (ASR) in adulthood, but only when the animals had been exposed to blood sampling under restraint prior to ASR testing [9,10]. The ASR is a characteristic sequential contraction of the skeletal musculature evoked by a sudden and intense acoustic stimulus [11]. In humans, increased basal startle is a marker for affective and anxiety disorders [12,13], whereas prepulse inhibition of the startle response (PPI) reflects an operational measure of sensorimotor gating and alterations in the PPI have been associated with schizophrenia and anxiety [14,15]. A number of studies have observed association between prenatal manipulation in the form of maternal stress or exposure to DEX and basal startle and/or PPI changes in the offspring, though the outcome has varied between studies [16–20]. Apart from the studies described above, maternal stress paradigms involving e.g. restraint or repeated variable stressors have not been associated with alterations in the basal startle reaction [21–23]. When startle testing included PPI, decreased [18] as well as increased [19]. PPI have been observed in the prenatally stressed offspring. In the studies that have assessed both the basal startle reaction and PPI after prenatal exposure to DEX, no effect on basal startle combined with limited effect on PPI [16] as well as blunted basal startle and increased PPI [17] has been reported. The latter study departed from the most commonly applied prenatal DEX protocol, that involves daily dosing in the range of 0.1 and 0.2 mg

\* Corresponding author. Centre for Psychiatric Research, Aarhus University, Skovagervej 2, 8240 Risskov, Denmark. Tel.: +45 77893524; fax: +45 77893549.

E-mail address: [wegener@dadlnet.dk](mailto:wegener@dadlnet.dk) (G. Wegener).

DEX/kg during the last week of gestation, coinciding with the appearance of glucocorticoid receptors in the fetal brain [24], by exposing the pregnant female rats to a very high dose of DEX (2 mg/kg) a few days early in gestation. Thus, some of the variability observed between these studies may at least partly be explained by differences in the choice of prenatal exposure window and dose levels. However, factors relating to the postnatal study design probably also play a role, e.g. age of the offspring when tested, prior behavioral assessments or timing of testing with regard to circadian phase. Some of the above studies tested ASR during the light phase of the circadian cycle whereas others tested the animals during the dark phase. In our previous work, increased basal startle only was observed in 6 month old prenatally manipulated female animals when tested during the light phase of their circadian rhythm [9,10] whereas decreased PPI only was observed in a study where similar animals were tested during the dark phase [25]. Based on these observations, we hypothesized that our prior observations of increased basal startle in female animals exposed to prenatal stress or DEX might have been influenced by diurnal phase [25]. This is especially relevant as perturbation of several physiological characteristics due to maternal stress have been shown to vary during the circadian cycle [26–29].

The hypothalamic–pituitary–adrenal (HPA) axis system is an essential part of the mammalian stress response, and activation of the HPA leads to release of glucocorticoids (corticosterone (CORT)) from the adrenal glands. CORT helps maintain basal activity of the HPA system and controls the sensitivity of the system's response to stress. CORT also exerts negative feedback on the HPA axis by way of glucocorticoid (GR) and mineralocorticoid (MR) receptors [3,30,31]. GR and MR are closely related intracellular CORT receptors but differ in their affinity for CORT and in the cellular effects they transduce [32,33]. Due to the 10 fold higher affinity of CORT for MR, MRs are fully occupied at basal levels of CORT whereas GRs become increasingly occupied with increasing CORT concentrations, such as those observed prior to the activity period of the light–dark cycle or in response to stress [34].

Regulation of the stress response also involves limbic areas such as the hippocampus [35,36]. Hippocampal neurons exert a tonic inhibitory control of CORT release by acting directly on the paraventricular nucleus of the hypothalamus (PVN) [37] and/or indirectly via neurons close to the PVN that regulate corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) release [38]. The hippocampal–HPA axis system is likely to be involved in the startle (ASR) response, because administration of CRH intensifies the ASR in rats [39–41]. The hippocampus contains CRH receptors (CRHR1 and CRHR2) as well as a peptide called corticotrophin releasing hormone binding protein (CRHBP) that acts as an endogenous modulator of the biological activity of CRH [42]. Furthermore, prenatal administration of the synthetic glucocorticoid DEX has been shown to alter the set point of the HPA axis [43] and to also affect hippocampal GR and MR mRNA expression [6,8,44].

The present study aimed to i) examine whether testing during the light versus the dark phase influences the effect of prenatal DEX on basal ASR, ii) determine whether changes in the expression of hippocampal glucocorticoid and mineralocorticoid receptors or related genes were associated with increased basal startle, and iii) investigate the generalisability of the model, regarding prenatal stress effects on the ASR, by performing it in a different strain and laboratory.

## 2. Materials and methods

### 2.1. Chemicals

DEX was obtained from Sigma-Aldrich, Denmark and was dissolved in 4% ethanol/isotonic saline.

### 2.2. Animals

Twenty-four time-mated young adult Sprague Dawley NTac:SD rats (Taconic M&B, Denmark) arrived at gestational day (GD) 7. Rats were randomly distributed singly to white plastic cages (27×43×18 cm, Scanbur, Denmark) with pine-bedding (Lignocel S8, Brogaard, Denmark). The cages also contained paper towels and a custom made metal “hiding box” (L×H×B:15×10×10 cm). Environmental conditions were automatically controlled with a 12-h light–dark cycle with lights on at 06:00 am. Food (Altromin Standard Diet 1324) and tap water were provided *ad libitum*. Clean cages and new bedding were provided once weekly. The animal welfare committee, appointed by the Danish Ministry of Justice, granted ethical permission for the studies. All procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Danish law regulating experiments on animals.

### 2.3. DEX exposure

From GD 14 to GD 21, time-mated rats were injected daily with DEX at a dose of 200 µg/kg s.c. [17,45,46] (N = 6) or vehicle (N = 6). The remaining 12 time-mated females were left undisturbed until delivery to be used as foster mothers. Injections were given during the dark phase of the light–dark cycle between 10:00 and 11:00 pm to maximize the glucocorticoid exposure of the animals.

### 2.4. Prewaning data

Nine treated time-mated females gave birth, as did nine of the non-injected time-mated females. Thus five CON and four DEX litters were used. Immediately after birth, 6–8 pups per dam were transferred to the 9 littering non-injected foster mothers. The animals were left undisturbed until weaning at 3 weeks of age except for general animal husbandry.

At postnatal day (PD) 21, up to four females per litter were weaned at random and housed in pairs based on similar prenatal exposure and postnatal testing. The animals were divided into groups determined by prenatal exposure (n = 20 CON, 17 DEX). The choice of female rats for this experiment was, in line with our previous studies, based on more women than men being diagnosed with anxiety disorders [1], yet most behavioral studies in this field use male rather than female animals. Therefore studies using female animals are highly warranted.

### 2.5. Post weaning investigations

Blood sampling and the first ASR test were performed during the light part of the diurnal cycle (between 08:00 am and 05:00 pm). The circadian cycle was then reversed (lights off 06:00 am) for the subsequent 3 weeks followed by a second startle test, which was conducted during the dark part of the diurnal cycle. Animals from the two prenatal exposure groups (CON and DEX) were equally distributed across the test days. It was decided to run the study in one set of offspring from one set of mother animals. The diurnal cycle would then be reversed for all tested animals, and, allowing sufficient time to acclimate to the reversed cycle, without compromising data [27]. This protocol ensured similar treatment of all offspring with regard to e.g. reversal of the circadian cycle and timing of husbandry during the circadian cycle.

#### 2.5.1. Postnatal acute stress by blood sampling under restraint

At 5 months of age, the animals were presented with an episode of postnatal stress by sampling tail blood three times from each of the CON and DEX animals as previously described [9,25] with minor modifications. Blood sampling under restraint was chosen as postnatal stressor, as a previous study show that exchange of blood sampling under restraint for another stressful procedure, i.e. forced

swim, did not produce comparable changes in startle performance [47]. Briefly, each animal was restrained in a clear acrylic restrainer, and blood was collected from a vertical incision in one of the tail veins (basal sample). After an additional 20 min of restraint, blood was collected again (stress sample). The rat was subsequently returned to its cage, and 1 h later restrained and sampled for the post stress sample. All animals were sampled on the same day. As previous work in our laboratory has revealed no difference in CORT response between CON and DEX rats [9,25] and concomitant sampling of many animals were probably associated with increased and skewed levels of disturbance both just before sampling of the basal sample and during the quiet period of “down regulation”, the blood was not stored for analysis.

## 2.6. ASR and PPI

The animals were tested for ASR at the age of 6–7 months using an SR-Lab™ SDI startle response system (SanDiego Instruments, INC. Europe). Testing was conducted as previously described [10,25]. At least 1 h prior to testing the animals were transferred to the experimental room. Throughout the startle protocol, white background noise (70 dB(A)) was delivered continuously inside the chambers from a 3.5" tweeter (model BT2, MG electronics, NY) 14 cm above the animal holder (a plexiglass tube, 8.8 cm diameter). The internal chamber light was switched on during testing. After a 5 min acclimatization period testing commenced with sessions lasting approximately 20 min and consisting of 45 trials. The startle eliciting stimulus consisted of a 40 ms broadband 120 dB(A) noise-burst. Each session started and ended with five 120 dB(A) startle trials followed by 35 test trials delivered in semi-randomized order (10 startle trials of 120 dB(A); 5 each of 4 prepulses (72, 74, 78 and 86 dB(A), respectively) + startle trials (denoted PPI72, PPI74, PPI78, and PPI86, respectively); 5 trials with no stimulus except background noise). Movement of the tube was registered for 100 ms after onset of the startle stimulus (sampling frequency 1 kHz), amplified, and the average response over 100 ms (AVG) was calculated. For each level of prepulse, AVGs were averaged and used for calculation of PPI. PPI was expressed as percent reduction in AVG compared to the average of the 10 middle startle trials using the following formula:  $\%PPI = 100 - ((AVG \text{ at prepulse} + \text{startle-trial}) / (AVG \text{ at startle trial}) * 100\%)$ .

## 2.7. Tissue collection

Four days after the second startle test all the rats were decapitated. The rats were transferred individually from the housing area to the laboratory and decapitated immediately (Harvard 55-0012 Small Animal decapitator, Scanbur AB, Sweden). The brain was extracted and immediately transferred to a cooled plate and divided along the midsagittal line. Based on prior randomization, one half hemisphere was frozen directly in pulverized dry ice, and the hippocampus from the other half was isolated and frozen directly before being transferred to Eppendorf PCR tubes and stored at  $-80^{\circ}\text{C}$ .

## 2.8. Western blot analysis

Half the animals had the left (L) hippocampus dissected and half the right (R) hippocampus. To exclude litter effects, only one L and one R hippocampus from each litter were used resulting in four groups of  $n = 4$  (CONL, DEXL, CONR, and DEXR). All procedures were carried out on ice according to the following protocol: Frozen hippocampi were weighed before being added to a mixture of cold homogenization buffer (20 mL/g tissue) (10 mM Tris-HCl, 300 mM sucrose and 2 mM EDTA; pH 7.4) and 1% protease inhibitor (Sigma-Aldrich, USA). The tissue was then homogenized (Tissue Tearor™, Biospec Products Inc., Bartlesville, OK, USA) at 30,000 rpm for 40 s before being centrifuged at 3600 rpm for 10 min at  $4^{\circ}\text{C}$  (Fisher

Scientific Accuspin™ Micro R, Pittsburg, PA). The resulting supernatant and pellet were further processed to generate the cytosolic and nuclear fraction, respectively. The cytosolic fraction was isolated by centrifuging the supernatant at 10,800 rpm for 15 min and aliquoting the final supernatant for storage at  $-20^{\circ}\text{C}$ . The nuclear fraction was isolated by resuspending the pellet in 200–400  $\mu\text{L}$  homogenization buffer followed by  $2 \times 10$  s bursts of sonication (Misonix, XL-2000, Qsonica LLC, Newton, CT, USA) and ultracentrifugation (Beckman Coulter Optima™ L-90 K, Fullerton, CA) at 18,000 rpm for 20 min at  $4^{\circ}\text{C}$ . The final supernatant comprised the nuclear proteins and was aliquoted and stored at  $-20^{\circ}\text{C}$ .

## 2.9. Western blot

The protein concentration in the individual samples was determined by protein assay in which serial dilutions of each sample were made with homogenization buffer and compared with a standard curve made with a known dilution range of Bovine Serum Albumin (BSA) (cat# BP 1605, Fisher Scientific, Pittsburgh, PA, USA). From each BSA standard and diluted sample, 10  $\mu\text{L}$  was pipetted in quadruplicate onto a 96 well microplate (Corning, NY, USA) and mixed with 200  $\mu\text{L}$  filtered Coomassie blue dye (20% Dye Reagent Concentrate, Bio-Rad Laboratories Inc, Mississauga, ON). The plates were read on a Biotek Synergy HT microplate reader (Biotek Instruments, Winooski, VT, USA). Only results from plates with a standard curve  $R^2 \geq 0.98$  were used.

Samples (20  $\mu\text{g}$ ) were mixed with 25% Laemmli loading buffer and boiled for 3 min. Stock Laemmli solution consisted of 1 mL Glycerol, 3 mL 10% Sodium dodecyl sulfate, 1.25 mL 0.5 M Tris base (pH 6.8) and 210  $\mu\text{L}$  of 1% Bromophenol Blue. The stock solution was reduced by 20%  $\beta$ -mercaptoethanol before use. Samples were loaded onto bis-acrylamide gels and the proteins separated by SDS polyacrylamide electrophoresis (Biorad Electrophor power supply, power pac HC, and Mini-PROTEAN 3 system, Bio-Rad Laboratories Inc., Mississauga ON) before being transferred to Immun-Blot® PVDF Membrane (cat# 162 0177, Bio-Rad Laboratories Inc.) on a Trans-Blot SD, semi dry transfer cell (Bio-Rad Laboratories Inc.). Blots were then blocked for 1.5 h in 5% milk powder in  $1 \times$  Phosphate Buffered Saline (PBS) solution (pH 7.4). All of the blocking and antibody incubation steps were performed with a 5% milk powder  $1 \times$  PBS solution derived from a stock solution of  $10 \times$  PBS (1 L) consisting of 80 g NaCl, 2 g KCl, 14.4 g  $\text{Na}_2\text{HPO}_4$  and 2.4 g of  $\text{KH}_2\text{PO}_4$  in ddH<sub>2</sub>O. Membranes were incubated overnight at room temperature (RT) with monoclonal Anti- $\beta$ -actin mouse antibody (Sigma-Aldrich Inc, USA) (1:2000) as a control for the amount of protein loaded. The following day, the membranes were washed ( $3 \times 5$  min) with ddH<sub>2</sub>O before being left to incubate 1.5 h at RT, with secondary anti-mouse IgG Peroxidase (Sigma-Aldrich Inc, USA) (1:20,000). Immunopositive bands were visualized using the Enhanced Chemiluminescence (ECL) plus western blotting system from Amersham (UK). Pictures of the bands were taken and a subsequent analysis was performed on a Kodak image station.

The membranes were then rinsed briefly with ddH<sub>2</sub>O before being left to block again for 1.5 h. Following this, they were left to incubate overnight with BuGR2 (1:500 in 5% milk powder,  $1 \times$  PBS solution) before being washed ( $3 \times 10$  min) with  $1 \times$  PBS and then left to incubate for 1.5 h at RT, with secondary anti-mouse IgG Peroxidase (Sigma-Aldrich Inc, USA) (1:10,000).

Visualization was performed as described above. All samples were run in duplicate. To quantify the bands, each was framed manually, as close to the band as possible, and the optical density was measured and subtracted from background density measured in an identically sized adjacent frame. To normalize the bands, the net GR band density was divided by the net  $\beta$ -actin band density.

BuGR2 has previously been found to recognize both the unactivated and the activated form of the native and denatured receptor [48].

## 2.10. Quantitative real-time polymerase chain reaction (real-time qPCR) analysis

Hippocampi from 4 CON and 3 DEX animals were used for the real-time-qPCR analysis. Tissue homogenization, RNA extraction, RNA characterization, cDNA synthesis, and real-time qPCR were carried out as described previously [49–52]. Briefly, hippocampi were homogenized in Lysis buffer (Applied Biosystems, CA, USA) with mixer-mill (Retsch) twice for 1 min (30 Hz/s). Total RNA was isolated using the ABI PRISM™ 6100 Nucleic Acid Prepstation (Applied Biosystems, CA, USA) following the manufacturer's instructions. Aliquots of the RNA solution were taken for both RNA quantification and quality analysis. The integrity of RNA and the RNA concentration were determined with RNA StdSens microfluidic chips using the Experion Automated Electrophoresis System (BioRad, CA, USA). RNA purity and concentration were determined by spectrophotometer (UV1650PC Shimadzu, Kyoto, Japan). Data on quality and purity of the extracted RNA were evaluated with Student's t-test. Purity of the 18s/28s rRNA ratios were between 1.2 and 1.5. Afterwards, RNA was reverse transcribed using random primers and Superscript III Reverse Transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. The cDNA samples were diluted 1:30 with DEPC water before being used as a qPCR template.

### 2.10.1. Real-time qPCR

Real-time qPCR reactions were carried out in 96-well PCR-plates using the Mx3000P (Stratagene, USA) and SYBR Green. Each SYBR Green reaction (10 µL total volume) contained 1× SYBR Green master mix (BioRad, CA, USA), 0.5 µM primer pairs, and 3 µL of diluted cDNA. The gene expression of GR, MR, CRHR1, CRHR2, CRHBP, and eight different reference genes (18s subunit ribosomal RNA (18s rRNA), beta-actin (ActB), Cyclophilin A (CycA), Glyceraldehyde-3-phosphate dehydrogenase (Gapd), Hydroxy-methylbilane synthase (Hmbs), Hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1), Ribosomal protein L13A (Rpl13A), and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (Ywhaz)) were investigated. The reference genes were selected as described by [53]. The primers were designed and tested as described by Elfving et al. The following forward and reverse primers were used: GR-forward: CACCCATGATCTGTCTAGTG, reverse: AAAGCCTCCCTCTGCTAACC; MR-forward: TAAGTTCCCCACGTGGTTC, reverse: ATC-CACGTCTCATGGCTTTC; CRHR1-forward: TGCCTTTTCTACGGTGCC, reverse: TGCAGTGACCCAGGTAGTTG; CRHR2-forward: GGAGCCC-TAGTGGAGAGACC, reverse: AGTGGCCCAGGTAGTTGATG; CRHBP-forward: GAGAGCCGTCTCACCAGAAG, reverse: GGTCACCGGATAAATGATGG; 18s rRNA-forward: ACGGACCAGAGCGAAAGCAT, reverse: TGTCAATCTGTCCGTGTCC (310 bp); ActB-forward: TGTCAC-CAACTGGGACGATA, reverse: GGGGTGTTGAAGGTCTCAA (165 bp); CycA-forward: AGCACTGGGGAGAAAGATT, reverse: AGCCACT-CAGTCTTGGCAGT (248 bp); Gapd-forward: TCACCACCATGGAGAAGGC, reverse: GCTAAGCAGTTGGTGGTGCA (168 bp); Hmbs-forward: TCCTGGCTTACCATTGGAG, reverse: TGAATTCCAGGTGAGGGAAC (176 bp); Hprt1-forward: GCAGACTTGTCTTCTTGG, reverse: CGA-GAGGTCC TTTTACCAG (81 bp); Rpl13A-forward: ACAA-GAAAAAGCGGATGGTG, reverse: TTCCGGTAATGGATCTTTGC (167 bp); and Ywhaz-forward: TTGAGCAGAAGACGGAAGGT; reverse: GAAGCATGGGGATCAAGAA (136 bp). Primers were obtained from DNA Technology A/S, Denmark.

### 2.10.2. Data analysis

For data normalization, we first measured mRNA levels for the reference genes. Stability comparison of the expression of the reference genes was conducted with the Normfinder software (<http://www.mdl.dk>) [54]. Ywhaz and 18s rRNA were determined to be the best combination in the hippocampus, and therefore values

for each individual were normalized with the geometric mean of these reference genes.

## 2.11. Statistical analysis

Startle data were analyzed by two-way ANOVA with Group (CON and DEX) and circadian period (light/dark) as factors, for PPI, with noise pressure level (72, 74, 78 and 86 dB(A)) as repeated measure. Since more than one pup per litter participated in each group, and thus introduced the risk for litter effects, pair wise comparisons were performed in a general linear model with pups nested within litter. Western blot data were analyzed by a one-way ANOVA with CONL, DEXL, CONR and DEXR as groups. Real time qPCR data were analyzed with a t-test.

## 3. Results

### 3.1. Basal startle

Overall statistical analysis on basal startle response revealed a significant effect of prenatal exposure (DEX:  $F(1,70) = 7.520$ ;  $P = 0.008$ ) and of circadian phase during testing (Phase:  $F(1,70) = 14.343$ ;  $P < 0.001$ ) (Fig. 1). This indicates that the basal startle reaction was altered in DEX offspring, irrelevant of phase, and in the dark compared to the light phase, irrelevant of prenatal exposure. Thus the prenatal exposure to DEX resulted in increased basal startle reactivity in both the light and the dark phases of the cycle.

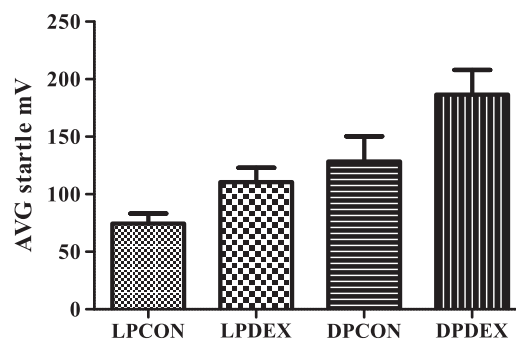
When analyzed separately for the light and the dark phases, basal startle was significantly increased in DEX compared to CON offspring during the light phase ( $[F(1,28) = 5.963$ ;  $P = 0.021$ ]; tested with pups nested within litter). Basal ASR also seemed increased in DEX compared to CON offspring when tested during the dark phase, although this difference did not reach statistical significance at  $p < 0.05$  ( $[F(1,28) = 3.462$ ;  $P = 0.073$ ]; tested with pups nested within litter).

### 3.2. PPI

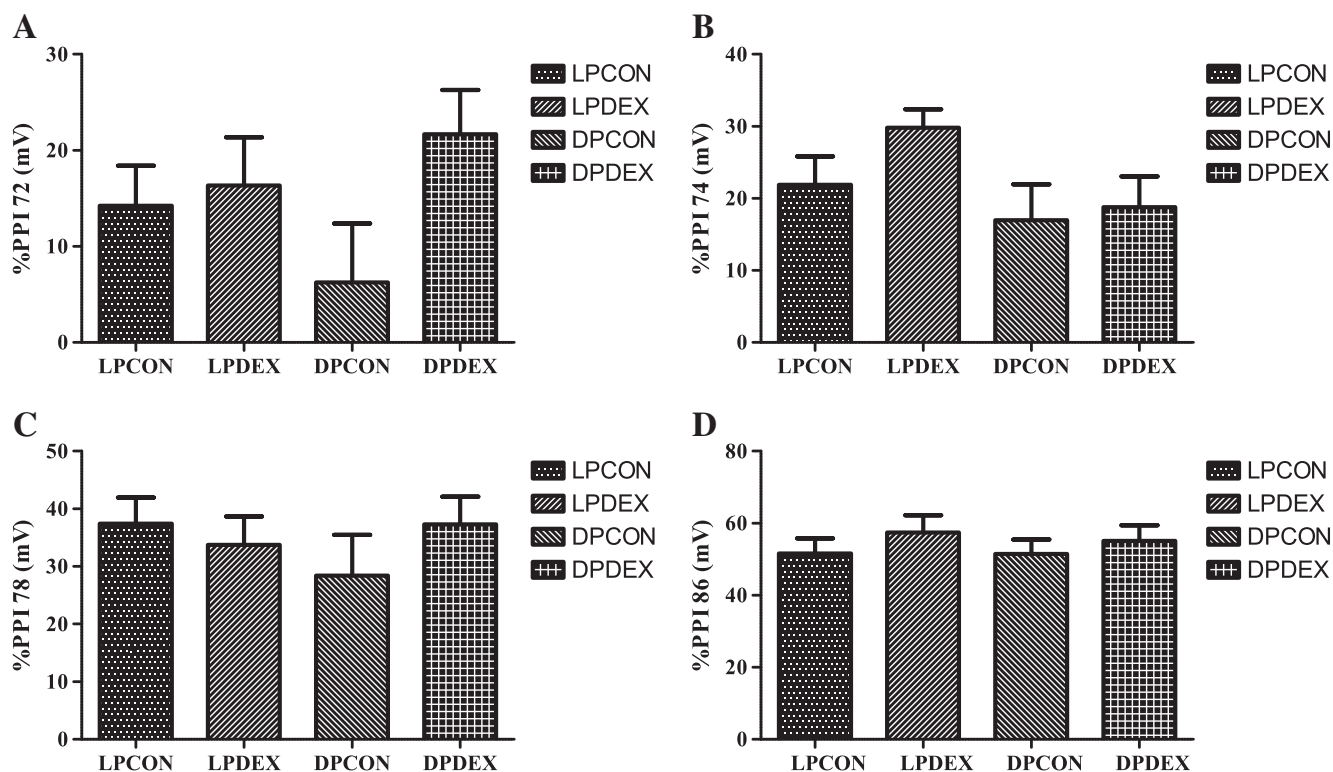
Data for PPI are presented in Fig. 2A–D. The overall statistical analysis did not indicate statistically significant differences due to prenatal exposure or time of testing.

### 3.3. GR protein expression levels in hippocampus

GR expression density in both L and R hippocampi did not vary statistically significantly between groups (Fig. 3).



**Fig. 1.** Mean basal startle response to the middle 10 120 dB pulses in female offspring tested during the light and dark phases of the circadian cycle with a 3 week acclimation period in-between. Testing was performed at age 6–7 months. LPCON = controls tested during light phase, LPDEX = rats prenatally stressed with dexamethasone and tested during light phase, DPCON = controls tested during dark phase, DPDEX = rats prenatally stressed with dexamethasone and tested during dark phase. Mean ± SEM,  $n = 17–20$ . \*Effect of DEX,  $P < 0.05$ .



**Fig. 2.** A–D — Mean average PPI72 (A), PPI74 (B), PPI78 (C) and PPI86 (D) in female offspring during the light and dark phase of the circadian cycle with a 3 week acclimation period in-between. Testing was performed at age 6–7 months. LPCON = controls tested during light phase, LPDEX = rats prenatally stressed with dexamethasone and tested during light phase, DPCON = controls tested during dark phase, DPDEX = rats prenatally stressed with dexamethasone and tested during dark phase.

Quantification of the optical density of the GR bands after normalization with  $\beta$ -actin revealed no statistically significant difference among the four subgroups (CONL, DEXL, CONR, and DEXR;  $F(1,12) = 0.976$ ;  $P = 0.343$ ) (Fig. 4).

#### 3.4. mRNA expression in hippocampus

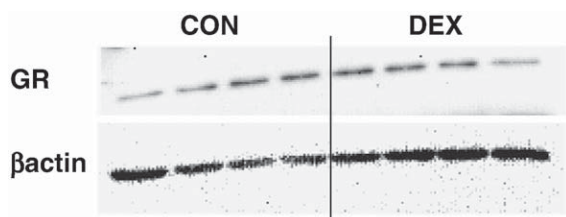
No statistically significant differences between the CON and the DEX-treated animals were found with respect to the 18s/28s rRNA ratio or the purity of the isolated RNA. The normalized mRNA levels for GR, MR, CRHR1, CRHR2, and CRHBP in the hippocampus are reported in Fig. 5A–E. For the first four genes, mRNA levels did not vary statically significantly between CON and DEX-treated animals, whereas CRHBP showed a trend towards increased expression in the DEX group compared to the CON group ( $t = 2.25$ ,  $df = 5$ ;  $P = 0.07$ ; Fig. 5E).

#### 4. Discussion

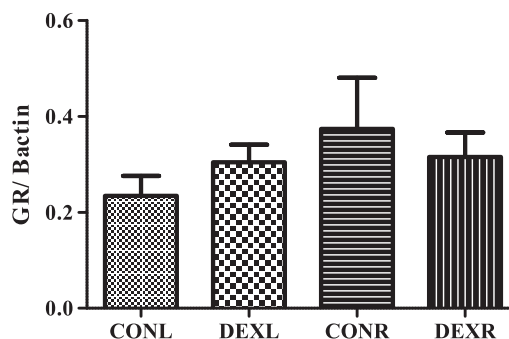
The main finding of this study is that offspring from DEX-treated mothers displayed elevated basal startle during the light phase of the

diurnal cycle, and that the pattern repeated itself during the dark phase. This replicates our earlier finding of increased basal startle in blood sampled animals, prenatally exposed to DEX and tested during light phase [9]. Furthermore, this replication of our earlier findings was performed in a new strain and laboratory.

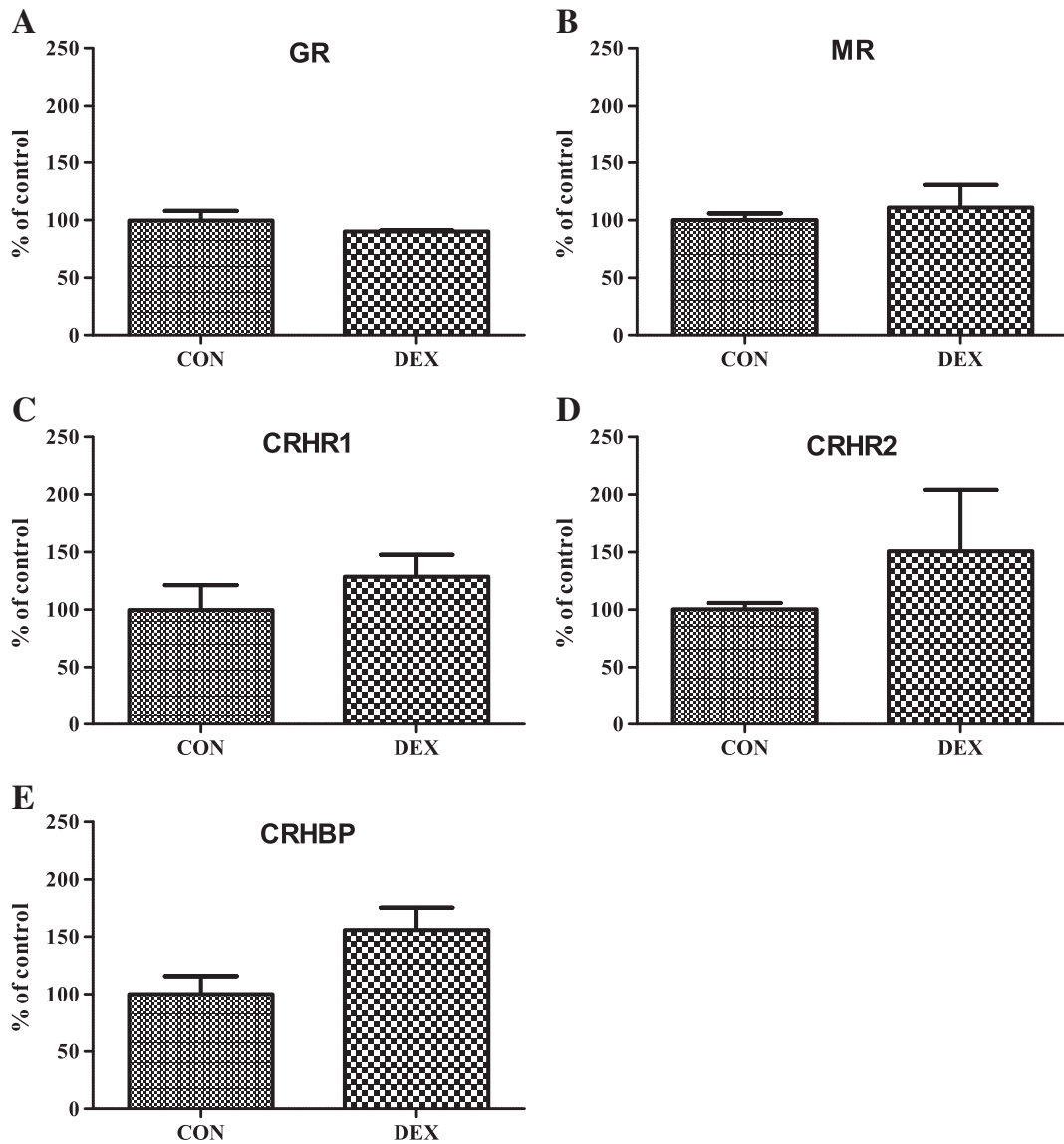
We have previously shown virtually identical increases in startle response during the light phase of the diurnal cycle in animals prenatally exposed to either CMS or DEX [9]. Increased fetal glucocorticoid exposure is possibly a common feature of both prenatal manipulations [9]. Testing during the light phase could be hypothesized to increase basal startle in animals prenatally exposed to glucocorticoids compared to CON because of a potentially increased stress load on susceptible animals since i) rats are nocturnal animals, and light seems aversive to them, hence the phenomenon of light enhanced startle [23,55], and ii) prenatally stressed rats, especially females, are more prone to anxiety [56]. The similarity between the startle response patterns during both diurnal phases indicates that the



**Fig. 3.** Western blot analysis of GR protein expression in homogenates of two representative hippocampi from the control (CON) group and the prenatal dexamethasone (DEX) group. Each sample was run in duplicate. Representative immunoblots probed with antibodies for GR and  $\beta$ -actin as an internal loading control are shown.



**Fig. 4.** Western blot analysis of GR protein expression compared with  $\beta$ -actin in homogenates of left (L) or right (R) hippocampi from the control (CON) and prenatal dexamethasone (DEX) group. Each sample was run in duplicate. Mean  $\pm$  SEM,  $n = 4$ .



**Fig. 5.** A–E – Messenger RNA samples from hippocampus of control (CON,  $n = 4$ ) and prenatal dexamethasone (DEX,  $n = 3$ ) groups were used for quantification of the expression levels of glucocorticoid receptor (GR) (A), mineralcorticoid receptor (MR) (B), corticotropin releasing hormone receptor1 (CRHR1) (C), corticotropin releasing hormone receptor2 (CRHR2) (D) and corticotropin releasing hormone binding protein (CRHBP) (E) using real-time qPCR. Values for each individual were normalized with the geometric mean of the reference genes *Ywhaz* and *18s rRNA*. Plotted data show mean group values + SEM of mRNA expression as % of control.

basal startle reaction was increased in DEX offspring, irrelevant of phase. The difference between CON and DEX animals only approached statistical significance during the dark period, however, which could be due to habituation to the startle procedure, i.e. three weeks between ASR testing may not have been sufficient time to overcome habituation completely [57]. That diurnal phase is not the sole determinant of increased startle in PNS animals is supported by other studies in which ASR testing of PNS animals have been conducted during the light phase without observation of increased basal startle [18,22].

CON and DEX animals showed no difference in PPI in the present study during either diurnal phase. This is in line with a previous observation from our laboratory in DEX exposed versus control animals [9]. We have, however, previously reported decreased PPI but no difference in the basal startle response in female rats exposed to prenatal CMS, compared with control animals [25]. In line with this, other studies, using either maternal restraint or repeated variable stress, have reported PPI changes but no difference in basal startle in the prenatally exposed offspring [18,19]. The present study used DEX as prenatal manipulation which might be speculated to differ in effect

from a more physical manipulation such as restraint or the complex variable exposures presented by repeated variable stress or CMS. Furthermore, the pharmacological properties of the primary hormone of the stress response, corticosterone, differ from the pharmacological stressor, DEX. As outlined in [16], increased plasma levels of corticosterone increase binding to both MR and GR. In contrast, DEX is a specific GR agonist. Furthermore, DEX crosses the placental barrier more readily than CORT [43] which potentially could indicate a higher exposure of the fetus with DEX administration. Our observation of an association between exposure to DEX in utero and changes in basal startle but not PPI in two studies makes it tempting to infer that DEX exposure somehow targets basal startle without affecting the PPI of startle, compared to “natural” stress. However, a previous study from our lab found similar ASR profiles (increased basal startle but no difference in PPI) in female offspring from dams exposed to either CMS or DEX, indicating a similar effect of the two manipulations on the regulatory mechanism of the ASR of the offspring [9]. Furthermore, a few other studies have reported weak PPI changes in offspring exposed to DEX in utero [16,47]. The observations of either PPI or basal startle alterations are intriguing as they seem to represent a

common, though not exclusive [17], outcome in studies of prenatal manipulation [9,10,16,18,19]. Even though basal startle and PPI may have to be viewed as two separate behavioral functions, there seems to be a strong interplay between them which may have great influence on the overall phenotype of the animal [47]. We speculate that the ASR phenotype of the animals (in the form of PPI and/or basal startle alterations) is determined by the degree of prenatal manipulations (timing and intensity) and postnatal experiences [25,47]. Previously, we have used prenatal exposure to CMS with the addition of daily allocations in a wire cage or saline injections combined with postnatal restraint [9,10]. These combinations of putative high intensity manipulations have, with the addition of the present results, now been associated with long term increased basal startle in both Wistar and Sprague Dawley rats and at two different laboratories [9,10]. Furthermore, effects persisted irrespective of cross-fostering. This replication of earlier findings under substantially different conditions strengthens the validity of our model [58], regarding effects of prenatal manipulation on ASR in female offspring. The persistence of effects after cross-fostering to some degree suggests that prenatal manipulation and not altered behavior of the dam founded the phenotype. The applied design of cross-fostering involved transfer of whole litters to foster dams, resulting in dams with either DEX treated or CON litters. Maternal behaviors are among others driven by stimulation from the pups (e.g. [59,60]), and are subject to alteration by adoption [61]. The applied procedure of cross-fostering was rather simple and does not dissociate completely the impact of the gestational treatment on pup physiology during fetal life from the impact of maternally induced effects after birth. Future studies attempting to dissociate pre- and postnatal events ought to apply a more elaborated design of cross fostering as e.g. described in [62].

In the work presented here, blood sampling under restraint served as the postnatal stressor similar to a previous study of ours, where an effect was found on ASR only in prenatally stress or DEX exposed animals that had been blood sampled [9,10]. Others have also reported the need for a “challenge” to bring out effects in PNS animals [63]. Importantly for our observations, part of the experimental procedure of ASR measurement resembles the blood sampling procedure, i.e. confinement in an ASR testing tube resembles restraint. This suggests that ASR effects in PNS animals are connected with aversive contextual similarities between blood sampling under restraint and the ASR test. In a recent study, we substituted the postnatal procedure of blood sampling under restraint with the forced swim test, which is a contextually different type of stressor [47]. Here, female rats exposed to DEX in utero showed increased immobility in the forced swim test but similar ASR behavior compared with the control animals. This indirectly supports our theory of a need for aversive contextual similarities between the postnatal stressful experience and the ASR test to show long term effects on ASR in prenatally manipulated animals. However, since the blood sampling under restraint procedure was not included in the forced swim study, it remains speculative whether the contextually more similar stressor would in fact have elicited increased startle [47]. Further studies should investigate whether prenatally manipulated animals are more aware of aversive contextual clues than controls and which cues are relevant to observe an increase of basal startle response in these animals.

In our previous studies, we found no change in CORT levels in PNS animals [9,10]. However, postnatal administration of CRH has been shown to intensify ASR in rats [39–41] even after adrenalectomy [64]. Therefore, the neural circuit regulating the ASR response may involve only some of the components of the HPA axis (i.e. CORT receptors; particularly GRs). Prenatal DEX [8,44] or restraint stress [7] during the last gestational week has been shown to reduce hippocampal GR and/or MR mRNA expression. In the present study, DEX exposure during the last gestational week showed no association with hippocampal GR

(mRNA and protein) and MR (mRNA) expression nor with CRHR1 and 2 (mRNA). This discrepancy could be due to at least three factors: timing of sampling, group sizes, and modification by postnatal experience. In a study by Koehl et al., binding capacity of hippocampal MR and GR were assessed in prenatally stressed offspring compared to non-stressed control offspring, at several time points across the 24 h cycle. Overall, binding capacity was reduced for both receptors in stressed compared to control offspring [27]. Although no diurnal fluctuations in overall binding capacity of either receptor were observed [27], visual inspection of the depicted data suggests that the difference was most prominent early compared to late in the light period. Although the measures of MR and GR were distinctly different in the two studies, it cannot be ruled out that sampling at another time point might have changed our outcome. Increasing group size and sampling at multiple points as in [27] might also have increased the possibility of detecting difference between the prenatal exposure groups. The lack of difference in GR, MR and CRHR1 and R2 availability should therefore be interpreted with caution and a larger study should be set up to test this further, preferably with multiple time points of sampling. Alternatively, postnatal restraint might have interfered with glucocorticoid receptor expression in animals exposed to prenatal DEX [6]. This study reports similar hippocampal GR and MR mRNA levels in animals treated prenatally with DEX and controls when assessed after postnatal restraint stress, whereas MR expression was reduced in prenatally DEX-treated offspring that had not been exposed to postnatal restraint [6]. Based on this study, our rats, all subjected to postnatal restraint would not be expected to differ in MR expression despite prenatal exposure to DEX. These findings indicate that not just prenatal glucocorticoid exposure but also postnatal experience influence adult GR and MR expression. Furthermore, since expression of these receptors was similar between PNS and CON animals, they do not appear to be directly involved in the increased startle response observed in animals exposed to DEX in utero.

We observed a tendency towards increased CRHBP mRNA expression in DEX-treated animals but not of CRHR1 and CRHR2, compared with CON animals. CRHBP has been shown to bind free CRH [65], but its exact role is not clear. Our data indicate that CRHBP might be associated with the altered startle response in animals exposed to DEX during gestation but a larger study is needed to confirm this. Further studies on the role of this peptide on stress responsivity are warranted.

In evaluation of behavioral studies the choice of gender should be considered. Female rats were chosen for this and our previous studies [9,10,25,47], as epidemiological data show women to have higher overall prevalence rates for anxiety disorders than men [1]. In animal studies of prenatal stress, gender differences with potential impact on stress responsivity are frequently noted [56]. Also the acoustic startle response varies with gender. Male rats generally display a higher basal startle levels than do female rats [16,22]. PPI has been shown to vary across the estrous cycle in female rats with reduced inhibition during proestrous compared to diestrous and estrous and to male rats [11]. Only a few of the cited studies have tested ASR in adult offspring of both males and females; two studies observed a selective effect on males of either increased startle [66] or increased PPI [16], and two studies reported no gender difference [19,22]. To draw conclusions regarding gender susceptibility with regard to prenatal manipulations and acoustic startle, more studies are needed.

Overall, the results suggest that our previous observations of changes in basal startle in rats exposed to CMS or DEX in utero were not solely dependent on diurnal phase. It should however be kept in mind, that the influence of phase cycle is discussed upon results obtained from two time points only, one in the light and one in the dark phase. For a more generalizable conclusion, i.e. to rule out that circadian phase influences prenatal stress effects on basal startle per se, it would be necessary to include more time points. We found no evidence of hippocampal GR and MR involvement in increased basal

ASR but a trend towards increased CRHBP in DEX animals. Nevertheless, a larger study is needed to corroborate our molecular findings, preferably with several sampling points during the circadian cycles. Future studies should investigate the role of CRH, CRH receptors and CRHBP and their relation to increased basal startle response.

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# Reduced Mobility But Unaffected Startle Response in Female Rats Exposed to Prenatal Dexamethasone: Different Sides to a Phenotype

S.L. Kjaer<sup>a, b</sup> G. Wegener<sup>a</sup> R. Rosenberg<sup>a</sup> K.S. Hougaard<sup>b</sup>

<sup>a</sup>Centre for Psychiatric Research, Aarhus University Hospital Risskov, Risskov, and <sup>b</sup>National Research Centre for the Working Environment, Copenhagen, Denmark

## Key Words

Anxiety · Mental illness · Forced swim test · Behavior · Environmental impact · Animal model · Dexamethasone · Pregnancy · Stress

## Abstract

An adverse fetal environment is strongly associated with behavioral and emotional development in later life, and environmental interactions with the genome are essential in the development of pathophysiology. This implicates that a genetic vulnerability or other predisposition may interact with the environment and stressful life events to trigger mental disease. The startle reflex is highly sensitive to fear and anxiety in humans and animals. Elevated startle magnitude has been proposed as a marker for neurodevelopmental disorders. We have recently established an animal model for possible development of anxiety, where female rats are exposed to two stressful life events, during prenatal life and as adolescents, respectively. A blood sampling procedure 3 months prior to startle testing has previously been found to increase basal startle, but only in prenatally stressed rats. As the experimental procedure of acoustic startle response (ASR) measurement resembles the aversive blood sampling procedure, this suggests that effects on ASR may be caused by aversive contextual similarities between blood sampling un-

der restraint and the ASR test. In the present study, postnatal blood sampling was replaced by another dissimilar stressful event. Animals exposed to a high prenatal glucocorticoid level (i.e. 150 µg dexamethasone/kg) were statistically significantly more immobile in the forced swim test (FST) than animals exposed to a lower level of dexamethasone (50 µg/kg) and control animals. Exposure to a novel contextual stressor at 3 months of age (FST) was unassociated with changes in basal startle. These data suggest, since the high prenatal dexamethasone group showed increased immobility in the FST but coped equally well with controls in the ASR, that the outcome of environmental influences is determined by the individual circumstances as different situations require different coping abilities in the same individual.

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## Introduction

A large number of human and animal studies show a strong association between an adverse fetal environment and behavioral and emotional development in later life [Abe et al., 2007; Maccari et al., 2003; Nagano et al., 2008; O'Connor et al., 2002; Tazumi et al., 2005; Van den Bergh et al., 2005]. Data from animal studies provide evidence that prenatal exposure to glucocorticoids restricts fetal

growth and suggest a role in programming the individual to adult disease [Hossain et al., 2008; Nagano et al., 2008; Newnham and Moss, 2001; Seckl, 2004; Seckl and Meaney, 2004; Weaver, 2009].

The acoustic startle response (ASR) is a characteristic sequential contraction of the skeletal musculature evoked by a sudden and intense acoustic stimulus [Koch, 1999]. In animals and humans, stimuli that induce fear and anxiety or administration of anxiogenic agents increase the startle response [Grillon, 2008]. In humans, elevated startle magnitude has been proposed as a marker for anxiety disorders, including anxiety and post-traumatic stress disorder, and possibly for major depressive disorder [Bakker et al., 2009; Grillon, 2002; Grillon et al., 2005]. Studies on anxiety and depressive disorders are important as these diseases constitute a substantial proportion of the global burden of disease [World Health Organization, 2008]. Also, an increase in studies using female animals is warranted in this research field as women have a higher burden of anxiety and depression than men [World Health Organization, 2008].

We have previously observed exaggerated startle in prenatally stressed or prenatally dexamethasone (DEX)-exposed female rats, but only in animals undergoing blood sampling under restraint prior to ASR testing [Hougaard et al., 2005a, b]. ASR testing involves confinement to a test tube. This resembles the experimental circumstances of blood sampling under restraint. In classic Pavlovian fear conditioning, a conditional stimulus is paired with an aversive unconditional stimulus in a novel context. After even a single pairing, animals will exhibit fear to the conditional stimulus, but also to the conditioning chamber and circumstances surrounding the conditioning episode [Anagnostaras et al., 2000]. Tazumi et al. [2005] observed increased baseline startle in prenatally stressed rats 1 week after exposure to light-potentiated startle, suggestive of contextual conditioning in these animals between the apparatus and the bright light. Therefore, it can be hypothesized that just one previous exposure to bright light during startle testing [Tazumi et al., 2005] or blood sampling in a bright room while being held in a restrainer in our lab [Hougaard et al., 2005a, b] could act as the unconditional stimulus/conditional stimulus/context pairing which induced the exaggerated startle in the subsequent startle test. This hypothesis is supported by studies using repeated exposure to a restrainer as a basis in post-traumatic stress disorder models [Harvey et al., 2003, 2004; Oosthuizen et al., 2005].

In the forced swim test (FST), the animals are tested for immobility, which is interpreted as either failure of persis-

tence in escape-directed behavior (i.e. behavioral despair) or development of passive behavior that disengages the animal from active forms of coping with stressful stimuli [Lucki, 1997]. In the present study, the FST was used both as a postnatal stressor and as a test in itself. This allowed us to investigate new aspects of our animal model, i.e. the association between FST and ASR and coping behavior.

Stressful experiences during gestation and early life have been hypothesized to enhance susceptibility for mental illness [Cottrell and Seckl, 2009; Fumagalli et al., 2007; Maynard et al., 2001] but a few studies in both humans and animals have shown an association between mild prenatal stress and protective or adaptive behavioral effects: mild to moderate levels of prenatal psychological stress were positively associated with mental development and advanced motor development in 2-year-old children [DiPietro et al., 2006], and prenatally stressed or DEX-exposed rats showed enhanced learning performance [Fujioka et al., 2001; Hougaard et al., 2005a] or lower stress-induced plasma corticosterone levels compared with controls [Van den Hove et al., 2005]. It has yet to be resolved why some individuals thrive under stressful conditions while others strive to survive. But it can be hypothesized that a system which during development has been exposed to mild levels of stress might become adapted to handling a stressor postnatally, whereas a naive system would be less successful.

Therefore, in the present work, we hypothesized that a high level of prenatal DEX would inhibit constructive coping whereas a lower dose of prenatal DEX might facilitate coping with postnatal stressful life experiences. The aims of this study were to examine (i) if prior exposure to a postnatal stressor, lacking contextual similarities with the ASR test, would induce changes in ASR in prenatally DEX-exposed animals and (ii) if prenatal exposure to a high versus a lower dose of DEX would result in different behavioral phenotypes.

## Materials and Methods

### *Chemicals*

DEX was obtained from Sigma-Aldrich, Denmark. The DEX used for animal experimentation was dissolved in 4% ethanol/isotonic saline.

### *Animals*

Sixty time-mated young adult rats (Wistars, HanTaC:WH, SPF) arrived at gestational day (GD) 4. The rats were randomly distributed pairwise to white plastic cages (Eurostandard III, 27 × 43 × 18 cm, Scanbur, DK) with pine-bedding (Lignocel S8, Brogaarden®, Denmark). The cages also contained nesting mate-

**Table 1.** Schematic overview of group exposures

Group	Prenatal exposure to DEX GD 14–21	FST 3 months	Acoustic startle test 6 months
CON			×
CONfst		×	×
DEXlow	×		×
DEXlowfst	×	×	×
DEXhigh	×		×
DEXhighfst	×	×	×

CON = Control; DEXlow = DEX 50 µg/kg/day; DEXhigh = DEX 150 µg/kg/day; FST = forced swim test.

rial (Enviro-Dri, Brogaarden®, Denmark), and environmental conditions were automatically controlled with a 12-hour light-dark cycle with lights off at 06.00 a.m. Food (Altromin Standard Diet 1324) and tap water were provided ad libitum. Clean cages and new bedding were provided twice weekly. The animal welfare committee, appointed by the Danish Ministry of Justice, granted ethical permission for the studies. All procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Danish law regulating experiments on animals (permission 2007/561-1396, C-schedule 1).

#### DEX Exposure

From GD 14 to 21, 40 rats were given daily s.c. injections with DEX, between 10.45 and 11.45 a.m., of which half received 50 µg/kg (DEXlow) and half 150 µg/kg (DEXhigh). The 20 control (CON) animals were injected s.c. with vehicle solution.

#### Pregnancy and Lactation Data

Forty-seven of the time-mated dams were observed with litters. The pups were weighed on postnatal day (PND) 3, and weaned at day 21.

#### Postweaning Investigations

At weaning, 1 female per litter was selected at random for each postnatal group (CON, CONfst, DEXlow, DEXlowfst, DEXhigh and DEXhighfst) and housed in nonsibling pairs of similar prenatal exposure and postnatal testing (table 1). The animals were housed under conditions as already described except for addition of aspen chewing bars (Brogaarden®, Denmark). From weaning, experimenters were kept unaware as to which prenatal exposure group the animals belonged. This was also in force during scoring of the FST data. Investigations were performed during the night phase of the diurnal cycle (dark phase) in the FST (see below) and the daylight phase (light phase) in the ASR test (between 8 a.m. and 5 p.m.). Animals from the different groups were equally distributed across the test days. Total group size: n = 32 CON, n = 24 DEXlow and n = 36 DEXhigh. Half the animals from each group were tested in the FST at 3 months of age.

#### Forced Swim Test

At 3 months, the animals from CONfst, DEXlowfst and DEXhighfst were tested in the FST as described previously [Porsolt,

1979; Porsolt et al., 1978] with minor modifications. Briefly, each rat was tested twice, 24 h apart. The animal was placed in a transparent cylindrical tank made from acrylic plastic (H: 55 cm, D: 24 cm). Each tank contained 38 cm of tap water (25 °C) which was changed between each trial. On day 1 (training phase), each rat was in the tank for 15 min, on day 2 (test phase) for 5 min. The animals were taken directly from the colony room before being tested by transfer of the home cage to an adjacent laboratory. After each session, the animals were dried in a towel and placed in an animal container heated by a thermal lamp until dry. On both days, swim sessions were recorded on video. Scoring consisted of determining the dominant behavior within 5-second intervals during the first 5 min of exposure time in each session. Behavior of the rats was divided into struggle, swim and immobility activity patterns according to Cryan et al. [2002].

The animals were not tested in the open field test prior to FST, as no difference in locomotion between prenatally DEX-exposed and CON animals was found in a pilot study in our laboratory.

#### Open Field Pilot Study

In a separate pilot study, female Sprague-Dawley offspring prenatally exposed to injections with 150 µg DEX/kg (DEX) daily from GD 14 to 21, according to the protocol described in the main experiment, were tested for locomotion in a square open field at the age of 4 months.

Twelve DEX and 12 CON were tested. The animals were observed for 5 min in a square open field (100 × 100 cm) situated on the floor in a bright room. The rat was placed in the center of the field, and the movements of the rat were recorded by video camera and analyzed for ambulation by Noldus Ethovision® XT, version 5. Between trials, the maze was cleaned with water.

#### ASR and Prepulse Inhibition (PPI)

All the animals were tested for ASR at the age of 6 months (table 1) using SR-Lab™ SDI startle response system (San Diego Instruments, Inc., Europe). Testing was conducted as previously described [Hougaard et al., 2005a; Kjaer et al., 2010].

At least 1 h before the test, the animals were transferred to the experimental room. Throughout the startle protocol, white background noise [70 dB(A)] was delivered continuously inside the chambers from a 3.5-inch tweeter (model BT2, MG Electronics, N.Y., USA) 14 cm above the animal holder (a Plexiglas tube 8.8 cm in diameter). The internal chamber light was on during testing. A 5-min acclimatization period commenced test sessions that lasted approximately 20 min and consisted of 45 trials. The startle-eliciting stimulus consisted of a 40-ms broadband 120 dB(A) noise burst. Each session started and ended with 5 120-dB(A) startle trials followed by 35 test trials delivered in semirandomized order: 10 startle trials of 120 dB(A); 5 each of 4 prepulses [72, 74, 78 and 86 dB(A), respectively + startle trials denoted PPI72, PPI74, PPI78, and PPI86, respectively]; 5 trials with no stimulus except background noise. Movement of the tube was registered for 100 ms after onset of the startle stimulus (sampling frequency 1 kHz), amplified, and the average response over 100 ms (AVG) was calculated. For each level of prepulse, AVGs were averaged and used for calculation of PPI. PPI was expressed as percent reduction in AVG compared to the average of the 10 middle startle trials: %PPI = 100 - [(AVG at prepulse + startle trial)/(AVG at startle trial) × 100%].

### Statistics

Analysis of variance (ANOVA) was used to analyze pregnancy and lactation data (table 2). In order to avoid litter effects, the litter was considered the statistical unit. The average body weight of pups within a litter was therefore used for statistical analysis and only one pup per litter was included in each of the experimental groups. Analysis of covariance (ANCOVA) was used to test for differences in maternal body weight gain and pup weight while controlling for litter size. Kruskal-Wallis One-Way ANOVA was used to test for litter size and gestational length. Post hoc comparisons were performed by ANCOVA. Forced swim data were analyzed by two-way ANOVA. Day 1 and day 2 were analyzed separately for average immobility or struggling within the first 5 min each day with group (CONfst, DEXlowfst and DEXhighfst) as factor.

Startle data were analyzed by two-way ANOVA, with prenatal exposure (CON, DEXlow or DEXhigh) and postnatal background (naive or FST) as factors. For PPI, data were analyzed separately for each prepulse intensity. When appropriate, Fisher's LSD was applied for pairwise comparisons. The accepted level of statistical significance was  $<0.05$  (SYSTAT Software Package version 12).

## Results

### Pregnancy and Lactation Data

Dams from the three groups were observed during pregnancy and lactation, and gestational data were recorded (table 2). No difference was found in litter size or gestational length between groups. Fewer DEXlow and CON dams gave birth to pups than DEXhigh. Of these pupless dams, 8 DEXlow and 3 CON showed no implantations, suggesting that impregnation had been unsuccessful. Maternal weight gain between GD 4 and 20 differed statistically significantly [ $F(2, 43) = 58.296$ ;  $p < 0.001$ ] with litter size as covariate. Pairwise comparisons showed that CON dams gained more weight than both DEXlow ( $p < 0.001$ ) and DEXhigh ( $p < 0.001$ ), and DEXlow dams gained more weight than DEXhigh ( $p = 0.006$ ). Offspring body weight at PND 3 (controlled for litter size) differed between groups [ $F(2, 43) = 29.507$ ;  $p < 0.001$ ]. Pairwise comparisons indicated a differentiation between all groups with increasing DEX exposure level inversely associated with pup weight ( $p < 0.05$ ) for all comparisons. At PND 20, an overall difference in body weight was still discernible [ $F(2, 42) = 6.080$ ;  $p = 0.005$ ], due to lower weight of DEXhigh pups than CON ( $p = 0.001$ ).

### Forced Swim Test

CONfst, DEXlowfst and DEXhighfst were tested in the FST at the age of 3 months. Behavioral activity was measured for the first 5 min each day. ANOVA of day 1 showed statistically significant scored differences in

**Table 2.** Pregnancy and lactation data

Endpoints	CON	DEXlow	DEXhigh
Number of litters <sup>1</sup>	16	12	19
Maternal weight gain GD 4–20, g <sup>2</sup>	98.4 ± 2.4 <sup>a</sup>	64.5 ± 3.7 <sup>b</sup>	51.7 ± 3.1 <sup>c</sup>
Gestation length, days	22.9 ± 0.1	23 ± 0.0	23 ± 0.1
Implantations	12.9 ± 0.8	12.8 ± 0.6	12.9 ± 0.5
Perinatal loss <sup>3</sup> , %	13.2 ± 5.9	17.0 ± 7.1	26.0 ± 5.9
Live pups per litter	11.8 ± 0.9	10.8 ± 1.1	9.6 ± 0.8
Female pups	6.5 ± 0.5	5.5 ± 0.8	4.7 ± 0.5
Pup weight (PND 3), g	7.5 ± 0.2 <sup>a</sup>	6.6 ± 0.2 <sup>b</sup>	5.2 ± 0.2 <sup>c</sup>
Pup weight (PND 20), g	31.7 ± 1.0 <sup>a</sup>	31.7 ± 0.8 <sup>a,b</sup>	30.4 ± 0.9 <sup>b</sup>

Values are mean ± SEM. Offspring parameters recorded on PND 3, unless otherwise stated. Pairwise comparisons were performed for maternal weight gain and pup weight, and values in the same row not showing a common superscript (<sup>a, b, c</sup>) are significantly different ( $0.001 \leq p \leq 0.05$ ).

<sup>1</sup> Initial number of mated females in each group was 20.

<sup>2</sup> Females with no live pups were excluded from this analysis.

<sup>3</sup> Percentage includes dams showing implantations but with no live pups at PND 7. Of the 13 dams with no pups, 11 showed no implantations (8 DEXlow, 3 CON), 1 DEXhigh had 10 implants and 1 CON had 1 implant.

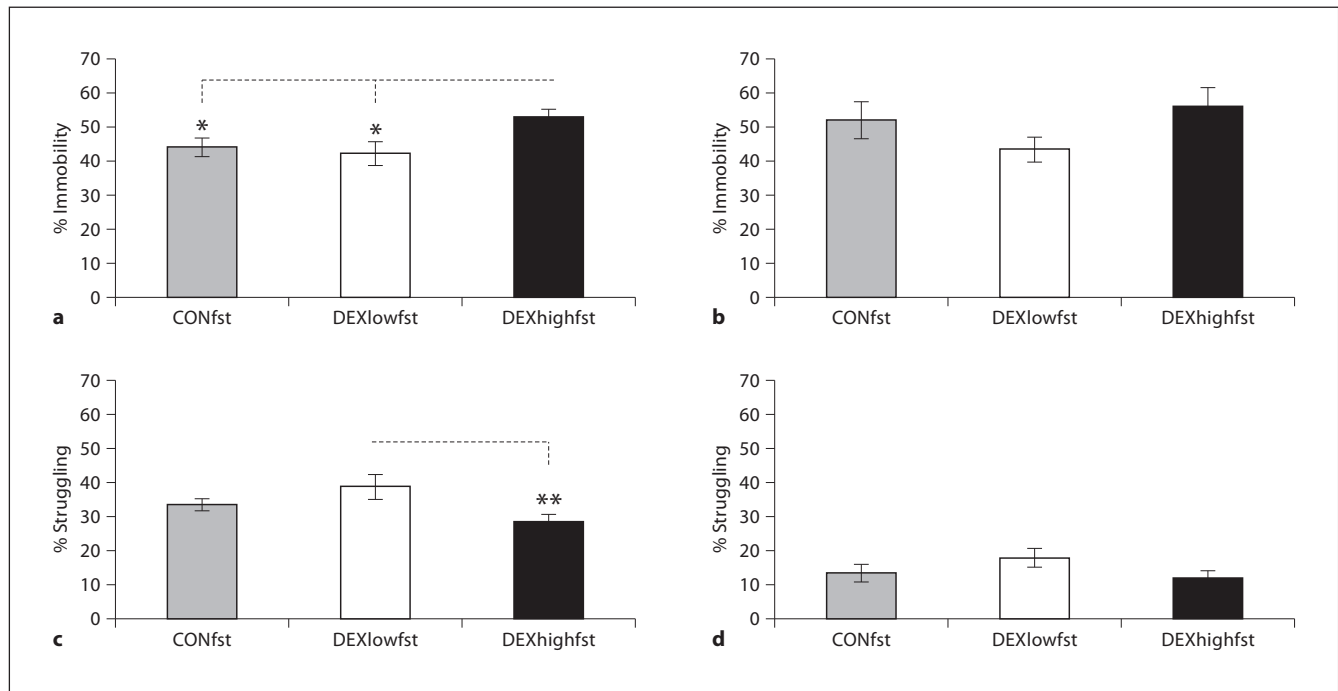
immobility and struggling between treatment groups [ $F(2, 43) = 4.419$ ;  $p = 0.018$ ] and [ $F(2, 43) = 4.833$ ;  $p = 0.013$ ], respectively]. Pairwise comparisons regarding effect of prenatal background demonstrated significantly increased immobility in DEXhighfst rats compared with CONfst ( $p = 0.021$ ) and DEXlowfst ( $p = 0.012$ ) (fig. 1a), and significantly less struggling in DEXhighfst compared with DEXlowfst ( $p = 0.003$ ) (fig. 1c). The same behavioral activity was visible on day 2, although not statistically significant (fig. 1b, d).

### Open Field Pilot Study

Female offspring prenatally exposed to injections with 150 µg DEX/kg were tested in the open field test at the age of 4 months. One-way ANOVA showed no difference in ambulation between prenatal treatment groups (DEX: 2,654 cm; CON: 2,500 cm) [ $F(1, 22) = 0.354$ ;  $p = 0.5$ ].

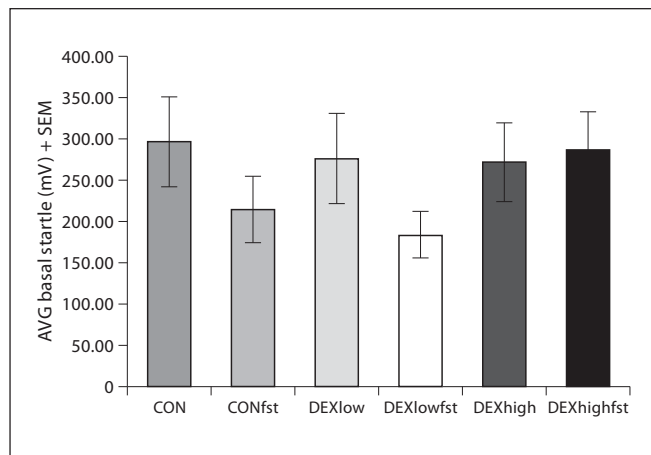
### Acoustic Startle Response

**Basal Startle.** At the age of 6 months, all rats were tested for ASR. Overall statistical analysis indicated no variation with prenatal exposure or postnatal FST. The average basal startle response during the middle 10 startle trials was similar in all groups (fig. 2). Comparable out-



**Fig. 1.** FST in female rats, age 3 months. **a** Percent time spent immobile during initial 5 min (day 1), group [F(2, 43 = 4.419; p = 0.018)]. **b** Percent time spent immobile during initial 5 min (day 2). **c** Percent time spent struggling during initial 5 min (day 1), group [F(2, 43 = 4.833); p = 0.013]. **d** Percent time spent struggling

during initial 5 min (day 2). CONfst = Control + FST; DEXlowfst = prenatal DEX 50  $\mu\text{g}/\text{kg}$  + FST; DEXhighfst = prenatal DEX 150  $\mu\text{g}/\text{kg}$  + FST. n = 16, 12, and 18 for CON, DEXlow and DEXhigh, respectively. Mean  $\pm$  SEM. \* p < 0.05, \*\* p < 0.01.



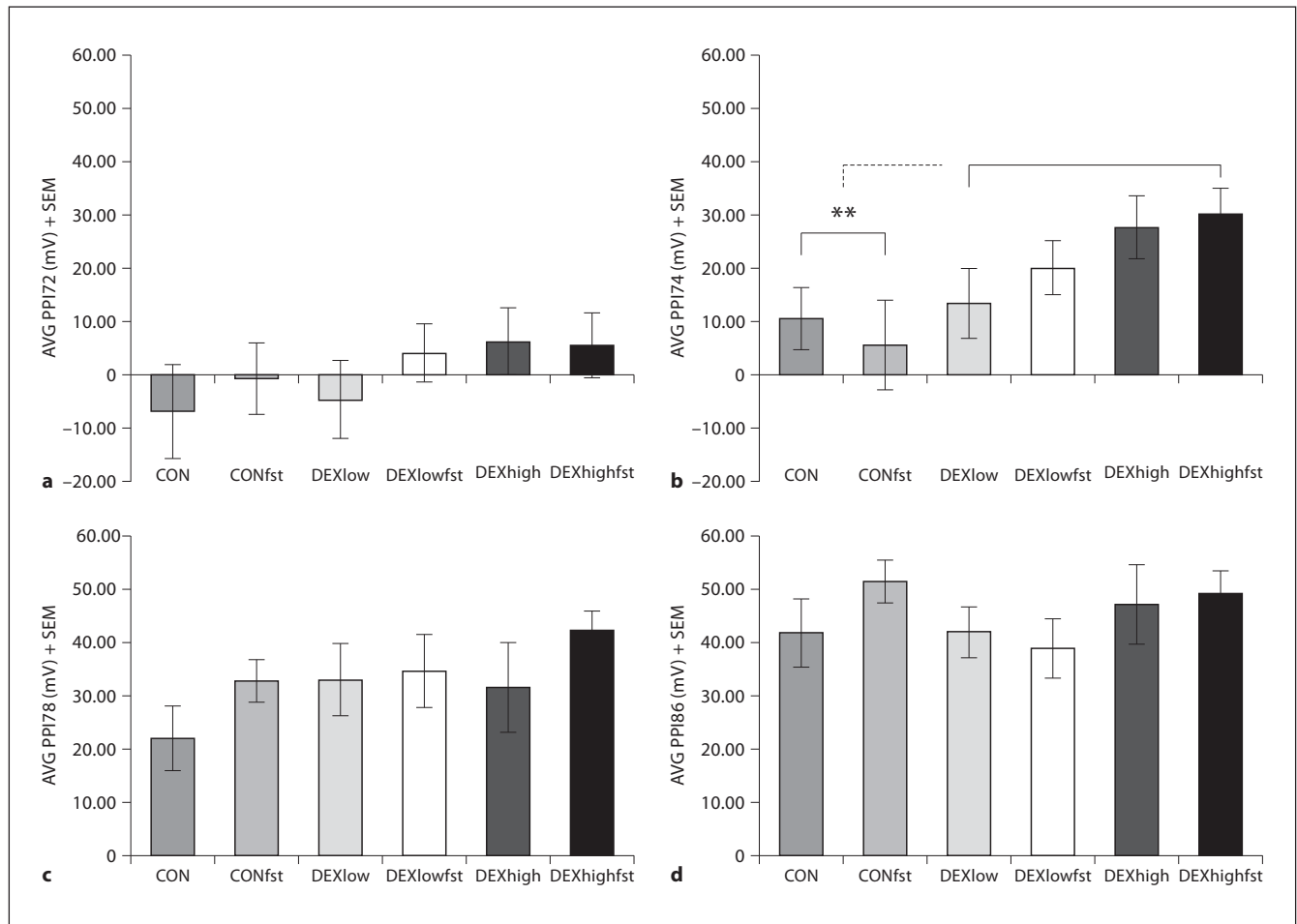
**Fig. 2.** Mean basal startle response to the middle ten 120-dB startle trials in female rats. CON = Control; CONfst = control + FST; DEXlow = prenatal DEX 50  $\mu\text{g}/\text{kg}$ ; DEXlowfst = prenatal DEX 50  $\mu\text{g}/\text{kg}$  + FST; DEXhigh = prenatal DEX 150  $\mu\text{g}/\text{kg}$ ; DEXhighfst = prenatal DEX 150  $\mu\text{g}/\text{kg}$  + FST. n = 16, 12 and 18 for CON, DEXlow and DEXhigh, respectively. Mean  $\pm$  SEM.

comes were registered for the 5 first and the 5 concluding startle trials [data not shown].

**Prepulse Inhibition.** PPI of the startle reaction to the 120-dB noise pulse was investigated for four levels of prepulses (72, 74, 78, and 86 dB). Statistical analysis showed that PPI72, PPI78 and PPI86 were similar in CON and DEX groups, but female offspring exposed to DEX in utero exhibited an increase of their mean average PPI74 independently of postnatal experience [F(2, 86 = 6.189); p = 0.003] (fig. 3).

## Discussion

There are two main findings of the present paper. First, prior exposure to FST was unassociated with changes in basal startle. Second, we observed a differential effect of DEX on immobility and struggling on day 1 of the FST together with a clear dose effect of DEX on pup weight at PND 3, which was normalized for DEXlow but not DEXhigh at PND 20.



**Fig. 3.** Mean average PPI72 (a), PPI74 (b), PPI78 (c), and PPI86 (d) in female offspring at age 6 months. CON = Control; CONfst = control + FST; DEXlow = prenatal DEX 50  $\mu\text{g}/\text{kg}$ ; DEXlowfst = prenatal DEX 50  $\mu\text{g}/\text{kg}$  + FST; DEXhigh = prenatal DEX 150  $\mu\text{g}/\text{kg}$ ; DEXhighfst = prenatal DEX 150  $\mu\text{g}/\text{kg}$  + FST.  $n = 16, 12,$  and  $18$  for CON, DEXlow and DEXhigh, respectively. Mean  $\pm$  SEM. \*\*  $p < 0.01$ .

The combination of prenatal stress (chronic mild stress) or prenatal DEX (100  $\mu\text{g}/\text{kg}$ ) with postnatal stressful blood sampling has been associated with increased basal startle in two previous studies from our group [Hougaard et al., 2005a, b]. Since positive association between blood sampling and increased startle was not present in the CON group, this study hypothesized that the prenatal stress or DEX-exposed rats may conceive a contextual link (restraint) between the two experiences. In line with this, Fujioka et al. [2001] reported enhanced ability to remember an association between an aversive stimulus and contextual clues in prenatally stressed rats. Furthermore, since increased startle can be interpreted as increased levels of anxiety or fear, contextual awareness

in prenatally stressed animals may correspond well with observation of anxious patients being sensitive to threatening contexts [Grillon, 2002]. In the present study, the combination of prenatal DEX exposure with FST was unassociated with changes in basal startle despite clear indications of affected behavior in DEXhigh rats in the FST. We suggest three different explanations for this: Firstly, the FST displayed no contextual similarities with the ASR test. Consequently, if the rats received no contextual warning of specific danger when placed in the ASR apparatus, this could explain the basal startle levels comparable with those of the CON animals. But since the blood sampling under restraint procedure was not included in this study, it remains speculative whether the contextu-

ally more similar stressor would have elicited increased startle in the present experiment, as observed previously [Hougaard et al., 2005a, b]. Secondly, the FST may have exerted a different stress exposure than blood sampling under restraint used as postnatal stressor in previous studies [Hougaard et al., 2005a, b; Kjaer et al., 2010]. The FST has been compared with restraint stress among other stress forms [Bowers et al., 2008; Mercier et al., 2003]. These studies used duration of increased HPA axis activity in the form of plasma or serum corticosterone (CORT) levels and core temperature changes (among others) as measures for stress intensity. Mercier et al. [2003] observed neither increase in plasma CORT nor in colonic temperature after 20-min swim stress, whereas 30-min restraint resulted in increased colonic temperature, when assessed during the dark period. Bowers et al. [2008] reported similar increases in circulating CORT after one exposure to either stress form but higher CORT values in the restraint than the FST group after repeated exposures. Yet, the latter study used mice and a duration of forced swim of only 2 min whereas the restraint procedure lasted 2 h per session. Despite the differences observed, exposure to the FST is undoubtedly a powerful stressor, but it cannot be excluded that the physiological and psychological responses might vary between the two procedures which confront the animals with different challenges (restriction of movement, pain and smell of blood versus swimming without escape). Thirdly, the animals were exposed to different doses of DEX (150 or 50  $\mu\text{g}/\text{kg}$ ) than in our previous study (100  $\mu\text{g}/\text{kg}$ ). It could therefore be speculated that the applied doses were less effective at eliciting increased basal startle than 100  $\mu\text{g}/\text{kg}$  DEX/kg. Nevertheless, results from our lab have shown that a prenatal dose of 200  $\mu\text{g}/\text{kg}$  DEX/kg in combination with postnatal blood sampling under restraint was associated with highly increased basal startle [unpubl. data], which makes it less likely that dose difference alone suffices to explain the unaltered startle.

Female offspring exposed to DEX in utero exhibited an increase in PPI74 independently of postnatal experience. This pattern was not repeated for the other levels of prepulse, so the possibility of a chance finding is highly likely. In line with this, a study by Hauser et al. [2006] also observed increased PPI to a specific stimulus level (84 dB) in prenatal DEX males. Since this result could not be replicated in a second experiment run by the same group, it was classified as a weak finding [Hauser et al., 2006].

Immobility during forced swimming has been interpreted as development of passive behavior that disengages the animal from active forms of coping with stressful

stimuli [Lucki, 1997]. In the present study, decreased mobility in the offspring from the DEXhigh group during their first exposure to the FST indicated a clear behavioral effect of the high prenatal DEX level, but the effect was only detectable on day 1. In a study by Welberg et al. [2001], the dams received either DEX treatments (100  $\mu\text{g}/\text{kg}/\text{day}$ ) throughout pregnancy (DEX1–3) or during the last third of pregnancy (DEX3). Here, the adult offspring from both groups showed either no difference (DEX1–3) or increased mobility (DEX3) in the FST. A higher dose of DEX (1 mg/kg/day) but given only on GD 18 and 19 gave no effect on FST [Oliveira et al., 2006]. A very recent study performed by Hauser et al. [2009] has yielded highly interesting results. They exposed pregnant dams to 100  $\mu\text{g}$  DEX/kg per day during the last week of gestation through the drinking water, and offspring were cross-fostered at birth to CON or DEX-exposed dams to enable separation of direct prenatal from indirect rearing dam-mediated effects of the treatment [Hauser et al., 2009]. The prenatal exposure was unassociated with changes in immobility, but rearing by a DEX-treated dam was associated with increased immobility in female rats [Hauser et al., 2009]. This importance of maternal care is supported by findings that maternal care/handling can affect the DNA methylation pattern [Weaver et al., 2004] and alter the stress responsiveness of the offspring [Meaney et al., 1988, 2007]. As decreased mobility was observed in DEXhigh and increased mobility was observed in DEXlow offspring, it would be interesting to study whether an association exists between DEX dose and maternal pup-directed behavior.

When considering changes in mobility as a behavioral response in the FST, the general locomotive drive of the animals should also be taken into account. Female rats exposed to 150  $\mu\text{g}/\text{kg}$  DEX/kg showed no difference in locomotion in the open field when compared with CON animals in our pilot study. Similarly, Hauser et al. [2009] observed no effect of prenatal or rearing dam treatment (CON or DEX) on distance moved in the open field test. In the study by Welberg et al. [2001], adult offspring from DEX1–3 and DEX3 groups showed reduced exploratory behavior in an open field, yet no difference (DEX1–3) or increased mobility (DEX3) was observed in the FST. When the locomotive behavior as such appears unaffected by treatment, this could indicate that the mobility changes in the FST tests is a coping response more than an expression of locomotive drive of the animals. However, since our open field results were obtained in different animals than those tested in the FST, reduced locomotive drive cannot be ruled out as a possible explana-

tion for the observation of reduced immobility in the DEXhigh group.

Welberg et al. [2001] observed increased mobility in (DEX3) animals. In line with this, our DEXlow offspring showed more struggling than the DEXhigh offspring. If increased mobility in the FST corresponds to increased coping and this increased mobility is facilitated by low levels of DEX, then it would be valuable to know what constitutes low glucocorticoid exposure: dose, timing or a combination. The dose of 50  $\mu\text{g}$  DEX/kg (GD 14–21) used in the present study might be too low for a clear effect of increased mobility as DEXlow offspring showed increased mobility compared with DEXhigh (150  $\mu\text{g}$  DEX/kg), but comparable levels with CON. Similarly, Nagano et al. [2008] used 50  $\mu\text{g}$  DEX/kg during the 3rd week of gestation and observed no difference in mobility between CON and DEX-exposed male offspring. Timing of the stressor is also clearly important. Mobility was increased in animals treated with 100  $\mu\text{g}$  DEX/kg during the last week of gestation compared with CON animals, whereas animals exposed during the entire gestation showed no difference in mobility [Welberg et al., 2001]. The importance of interaction between dose and timing is in accordance with the concept of programming and neurodevelopment. Different cells and tissues are sensitive at different times, so the influences of environmental challenges will have distinct effects, depending not only on the challenge involved but also upon its timing [Seckl, 2004]. More studies are needed in this area to elucidate what might constitute a beneficial stress exposure. It should be noted, however, that coping ability was test-specific in our study, i.e. a difference between groups was observed in FST but not in basal startle, which makes it less clear-cut to distinguish between adaptive and maladaptive phenotypes.

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Growth restriction in the offspring after glucocorticoid administration in the dam is a common finding [Newnham and Moss, 2001]. We observed a clear dose effect of DEX on pup weight at PND 3, normalized by PND 20 for DEXlow but not DEXhigh. This further supports the notion that the offspring were differentially affected by the different DEX concentrations.

In conclusion, we observed a differential effect of DEX on immobility and struggling in FST on day 1 but no effect on basal startle in the same animals. Possibly, contextual clues play a role for subsequent induction of increased basal startle reactivity in prenatally DEX-exposed rats, but further studies are required to clearly determine which cues are relevant to observe an increase of basal startle response in animals exposed to DEX in utero. Also, different situations require different coping abilities in the same individual, and distinguishing between harmful and adaptive stressful exposures may not be apposite. Instead, the outcome of early-life experiences may be determined by the degree of ‘match-mismatch’ between prenatal exposures and future environment [Oitzl et al., 2009].

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## Gestational chronic mild stress: Effects on acoustic startle in male offspring of rats

K.S. Hougaard<sup>a,\*</sup>, K.R. Mandrup<sup>c</sup>, S.L. Kjaer<sup>a,b</sup>, I.B. Bøgh<sup>d,1</sup>, R. Rosenberg<sup>b</sup>, G. Wegener<sup>b</sup>

<sup>a</sup> National Research Centre for the Working Environment, Lersø Parkallé 105, DK-2100 Copenhagen Ø, Denmark

<sup>b</sup> Centre for Psychiatric Research, Aarhus University Hospital Risskov, Skovagervej 2, DK-8240 Risskov, Denmark

<sup>c</sup> National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark

<sup>d</sup> Veterinary Reproduction and Obstetrics, Faculty of Life Sciences, University of Copenhagen, Dyrlægevej 68, DK-1870 Frederiksberg C., Denmark

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### ABSTRACT

An increasing number of scientific studies indicate that maternal stress during pregnancy influences fetal development of the nervous system and thereby the behavioural phenotype. We have previously reported attenuated prepulse inhibition (PPI) of the startle reaction in adult female rats derived from dams exposed to chronic mild stress (CMS) during gestation. In humans, decreased PPI has been reported to be associated with anxiety. Because of its potential translational value across species, the modulation of startle reactivity may be a useful tool in examining altered emotional reactivity following prenatal insults. The present study aimed at investigating whether prenatally stressed male offspring would display altered startle phenotype. Stress was induced by maternal gestational exposure to alternating procedures, i.e. CMS. At the age of 3 months, half of the offspring were blood sampled under restraint. At the age of 6 months, i.e. three months later, all animals were tested in the acoustic startle and the light enhanced startle (LES) paradigm. Control and CMS male offspring showed similar basal startle and LES levels. Maternal gestational exposure to the relatively mild, variable paradigm of stressors affected the PPI response pattern in male rats. In prenatally manipulated males, the PPI response differed statistically significantly, depending on prior exposure to an episode of postnatal acute stress (blood sampling under restraint). In contrast, the PPI response in control males was unaffected by this postnatal experience. The present work supports the hypothesis that the maternal environment is a long-term determinant of phenotypic differences in sensitivity to stressors.

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### 1. Introduction

Increasing amounts of scientific evidence indicate that alterations of the intrauterine environment predispose the offspring for development of disease in later life. The fetal programming hypothesis states that the uterine environment may interact with fetal development, imposing a permanent effect on the resulting phenotype (Barker, 1998). Recent findings indicate that maternal stress during pregnancy influences fetal development of the nervous system and thereby the behavioural phenotype of the offspring (Van den Bergh et al., 2005; Weinstock, 2008).

In a series of studies in our laboratory, female offspring from dams undergoing stress or exposure to glucocorticoids during ges-

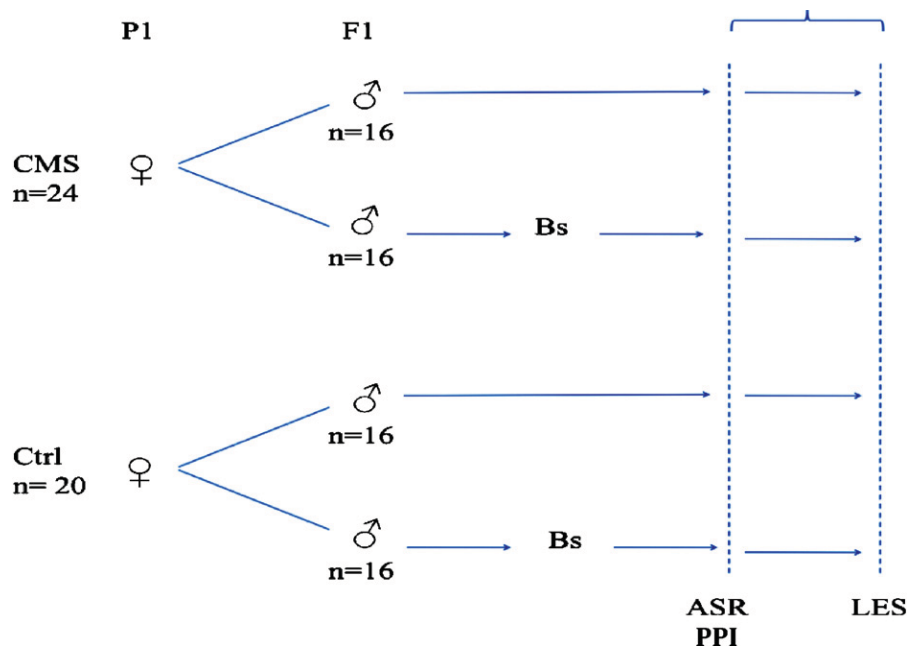
tation displayed changes in the acoustic startle reaction when compared to control female offspring (Hougaard et al., 2005a, 2005b; Kjaer et al., 2010b). This change seemed to depend on blood sampling under restraint months before startle testing (Hougaard et al., 2005b; Kjaer et al., 2010a). Acoustic startle arises as a fast reflexive contraction of skeletal musculature upon a strong unexpected acoustic stimulus (Koch, 1999). The reaction may be attenuated by a prepulse-stimulus (pre-pulse inhibition, PPI), reflecting a measure of sensorimotor gating (Braff et al., 1992). The observations of changed startle reactivity are much in line with the hypothesis that disturbing circumstances during fetal life may leave the offspring more vulnerable to events later in life (Oitzl et al., 2009). Startle is highly sensitive to fear and anxiety in both humans and animals (Grillon, 2002; Grillon, 2002, 2005) and decreased PPI has been reported to be associated with anxiety in humans (Franklin et al., 2009; Ludewig et al., 2002). Furthermore, changes in PPI has been demonstrated to be reflective of sensory gating deficits in schizophrenia (Braff et al., 2001; Kumari et al., 2008; Li et al., 2009). Because of its potential translational value across species, modulation of startle reactivity may be very useful in examining altered emotional reactivity following prenatal insults (Bijlsma et al., 2010).

*Abbreviations:* ASR, acoustic startle reaction; CMS, chronic mild stress; GD, gestation day; LES, light enhanced startle; PND, postnatal day; PPI, prepulse inhibition.

\* Corresponding author. Tel.: +45 39165217; fax: +45 39165201.

*E-mail addresses:* ksh@nrcwe.dk (K.S. Hougaard), kaman@food.dtu.dk (K.R. Mandrup), slk@nrcwe.dk (S.L. Kjaer), ibbh@novonordisk.com (I.B. Bøgh), raben@dadl.dk (R. Rosenberg), wegener@dadl.dk (G. Wegener).

<sup>1</sup> Present address: Novo Nordisk A/S, NovoNordisk Park, DK-2760 Måløv, Denmark.



**Fig. 1.** Study design. Pregnant rats (P1) were exposed to either chronic mild stress (CMS) or control conditions (Ctrl), on GD 9–21. At weaning, two males per litter (F1) were selected for further testing. One was blood sampled (Bs) at the age of 3 months. At approximately 6 months of age, the startle reaction was tested in all weaned males, first basal startle and prepulse inhibition (ASR), and one month later light enhanced startle (LES).

Epidemiological data indicate that women have a higher overall prevalence rate for anxiety disorders compared to men (Pigott, 2003; World Health Organization, 2008). Similarly, in animal studies the female gender often displays a relatively stronger anxious and depressive phenotype than males (Weinstock, 2007). Accordingly, our previous startle studies were primarily conducted in female offspring. Studies of prenatal stress only rarely investigate effects in both genders, even if genders may differ in the sensitivity of developing brain areas to stress hormones (Weinstock, 2007). The present study therefore aimed at investigating whether the startle phenotype of male offspring would be influenced by maternal exposure to chronic mild stress (CMS) during gestation. Prenatal manipulation was combined with blood sampling under restraint as postnatal stressor, followed by test of basal startle and PPI in adult life, similarly to investigations performed in female offspring previously (Kjaer et al., 2010b). In addition, male rats were tested for light enhanced startle (LES) since this specific test has been shown to be sensitive to detect alterations induced by prenatal stress in male rats (Tazumi et al., 2005).

## 2. Materials and methods

The study design is outlined in Fig. 1. The procedure is identical to that applied to the female siblings described in (Kjaer et al., 2010b), but for the concluding test of LES.

### 2.1. Animals

Upon arrival at gestation day (GD) 3, time-mated young adult nulliparous Wistar rats, Han TaC: WH, SPF (Taconic M&B, Denmark) were housed pair wise in white plastic cages (27 cm × 43 cm × 15 cm) with pine bedding (Lignocel S8). Environmental conditions were automatically controlled with a 12 h light/12 h dark cycle (lights on at 7 p.m.). Food (Altromin Standard diet 1324) and tap water were provided ad libitum. Cages were cleaned and new bedding provided twice weekly. All procedures complied with the EC Directive 86/609/EEC and with the Danish law regulating experiments on animals (permission no. 2007/561-1396 C1).

### 2.2. Maternal exposure

On GD 4, the animals were weighed and allocated to 2 groups with similar weight distributions [Ctrl 195.3 ± 1.6 g (SEM) and CMS 195.0 ± 1.3 g (SEM)]: 20 served as controls (Ctrl) and 24 were subjected to CMS at GD 9–21. CMS is a schedule of chronic stress, where various relatively mild stressors are presented in a random schedule

(detailed description in (Hougaard et al., 2005b)). Each stressor was applied once or twice during the period of exposure, and the procedures were evenly distributed throughout the period and with respect to the light and dark phases of the animals. The stressors employed were space restriction by placing the rats in a smaller cage (25 cm × 20 cm × 14 cm; 1 × 8 h and 1 × 16 h); food and water deprivation for 2 × 8 h; empty water bottle (1 h); wet bedding for 1 × 9 h and 1 × 16 h); crowding for 2 × 8 h; new partner for 1 × 24 h; isolation (1 × 16 h); tilted cage (45°; 1 × 8 h and 1 × 16 h) and placement in a wire cage (1 × 8 h and 1 × 16 h). Body weights were recorded at GD 7, 10, 13, 15, 17, 19, and 21. After termination of exposures at GD 21, the females were singly housed. All time-mated dams gave birth, most on GD 22–23. Litter size and gender were registered on postnatal day (PND) 3. Body weights of the progeny were recorded on PND 3, 10 and at weaning on PND 22. The dams were sacrificed by decapitation the day after weaning, and were examined for the number of uterine implantation sites.

At weaning, two males per litter were randomly selected for further testing and housed in non-sibling pairs under the same conditions as described above. One male of each litter pair was blood sampled at the age of 3 months. All males were startle tested at 6 months of age, resulting in a total of four different treatment groups: Controls with or without postnatal blood sampling (Ctrl+ and Ctrl–) and animals originating from dams exposed to CMS during pregnancy with or without postnatal blood sampling (CMS+ and CMS–).

### 2.3. Plasma corticosterone

For blood sampling at the age of 3 months, one rat at a time was transferred by hand to an adjacent laboratory and restrained (immobilizer IM/OH Scanbur A/S, Denmark). Two vein blood samples were collected from a vertical incision in the tail, with 20 min interval before returning the animal to the cage. One hour later, a final blood sample was taken immediately after a second restraint, i.e. 80 min after the initial sample. Blood samples one and three were collected within 2 min after removal of the animal from the cage. Cage mates were sampled on different days. The blood was collected in Microtainer® tubes with K<sub>2</sub>EDTA (Becton Dickinson no. 5973). After centrifugation, plasma was separated, aliquoted and stored at –80 °C until analysis. Corticosterone level in plasma was determined by competitive radioimmunoassay as described previously (Kjaer et al., 2010b; Pedersen et al., 2000).

### 2.4. Acoustic startle

At the age of 6 months, all males were tested for acoustic startle reaction (ASR) and prepulse inhibition (PPI) in two chambers (San Diego Instruments, San Diego, CA, USA) with 70 dB(A) white background noise as described in (Hougaard et al., 2005a). A piezoelectric accelerometer transduced displacement of test tubes (∅ 8.2 cm) in response to the movement of the animal. A test session proceeded as follows: 5 min acclimatization; 5 startle stimuli of 120 dB; 35 randomized prepulse-, startle- and no stimuli (10 startle stimuli of 120 dB(A); 5 each of 4 different levels of

**Table 1**  
Pregnancy and lactation data.

End point	Control	CMS
Time-mated females in study	20	24
Time-mated females giving birth	20	24
Gestation length	22.7 ± 0.1	22.9 ± 0.1
Maternal weight gain GD 7–21 (g) <sup>*</sup>	81.5 ± 2.9	74.1 ± 3.5
Litter size	9.5 ± 3.3	8.6 ± 3.1
Implantations	10.6 ± 0.5	10.4 ± 0.7
Implantation loss (%)	11.2 ± 2.7	18.0 ± 4.0
Pup weight, PND 3 (g)	8.18 ± 0.2	8.13 ± 0.2
Pup weight, PND 10 (g)	18.8 ± 0.5	18.9 ± 0.7
Pup weight, PND 22 (g) <sup>*</sup>	42.8 ± 1.0	44.8 ± 1.6

Values are mean ± SEM.

<sup>\*</sup> 0.05 < *P* < 0.1.

prepulses plus startle stimulus [72,74, 78 and 86 dB(A), denoted PPI72, PPI74, PPI78 and PPI86], and 5 trials with background noise only) followed by 5 final startle stimuli of 120 dB(A). Inter-trial intervals (the intervals between the individual stimuli) were randomized between 10 and 20 s (mean 15 s). The average amplitude (AVG) of the startle response was calculated as the mean of the 5 initial (AVG<sub>initial</sub>), 10 middle (AVG<sub>middle</sub>), and 5 last (AVG<sub>final</sub>) stimuli for each of the exposed groups.

PPI was expressed as percent reduction in the averaged five AVGs for each prepulse intensity compared to the average of the 10 middle startle trials: %PPI = 100 – [(AVG at prepulse + startle trial)/(AVG at startle trial)] × 100%. Animals were tested twice (Week 1 and Week 2) with 1 week between sessions to test for persistency of effect. The internal chamber light (~80 lux) was on during testing.

Animals were tested for LES as described in (Tazumi et al., 2005), with minor modifications, one month later. This delay was interposed to separate the test sessions in time and thereby avoid overt carry over habituation from the first two sessions to the LES session. The LES test session started with 5 min of acclimatization in the startle chamber with lights off followed by a sequence of 40 sound stimuli of 100 dB. Then lights were turned on and the animals presented with 40 identical stimuli in bright light (~1200 lux). All stimuli were emitted with an interval of 30 s. The 80 stimuli were averaged over 8 blocks of each 10 stimuli, i.e. 4 blocks in darkness and 4 blocks in light. The initial 3 blocks (30 stimuli) are considered habituation, the 4th block in darkness represents baseline level, and block 5–8 represents the test phase.

It happens, although rarely, that a male rat startles so powerfully that it dislocates the test tube from its socket whereby monitoring terminates too early. Data from these runs were left out of the final data compilation.

### 2.5. Statistics

The litter was considered the statistical unit in order to avoid litter effects. Litter size, sex distribution, and loss of implantations were analyzed by the Mann Whitney *U* test. Analysis of covariance (ANCOVA) was applied to test for differences in maternal body weight gain, while controlling for litter size. Offspring lactational body weight development was also analyzed by ANCOVA, with days as repeated measure. Post hoc comparisons were likewise performed by ANCOVA. Startle data were analyzed using a three way ANOVA, with Group (Ctrl and CMS), Blood sampling (±) and Week as factors. The accepted level of significance was *P* < 0.05. Habituation of the basal startle response over the initial 5 trials was analyzed with two way ANOVA (Group × Blood sampling) with impulse (1–5) as repeated measure. PPI data were analyzed separately for each level of prepulse. For LES, Group (Ctrl and CMS) and Blood sampling (±) were between-subject factors, and Block 4–8 served as within-subject (repeated) measure. Pairwise comparisons were performed by the protected Fisher's least-significant-difference test (Fisher's LSD) (Holson et al., 2007). Plasma corticosterone concentrations at age 3 months were similarly compared between the 4 treatment groups, but with Sample (basal, stress, and post-stress) as the repeated measure. All analyses were performed in SYSTAT v. 9.

## 3. Results

### 3.1. Gestational data

There were no statistically significant differences associated with gestational exposure related to sex ratio, litter size, or loss of implantations (Table 1). Statistical analysis of maternal weight gain during gestation (ANCOVA with litter size as covariate) indicated a major influence of litter size [ $F(1,41) = 49.481$ ;  $P < 0.001$ ], while exposure to CMS tended to depress weight gain compared to controls [ $F(1,41) = 3.354$ ;  $P = 0.074$ ; Table 1]. Pair wise comparisons (ANCOVA) revealed no main effect of CMS on separate days. Analysis of offspring body weight development during lactation

revealed effect of litter size [ $F(1,38) = 99.519$ ;  $P < 0.001$ ], but no main effect of exposure. Within subjects, there was a main effect of postnatal day [ $F(2,76) = 1147.083$ ;  $P < 0.001$ ] reflecting the general weight gain during lactation. Postnatal days interacted with litter size [ $F(2,76) = 130.036$ ;  $P < 0.001$ ] as well as CMS [ $F(2,76) = 5.134$ ;  $P = 0.008$ ]. Pair wise comparisons (ANCOVA) revealed a tendency towards enhanced body weights in CMS offspring compared to control offspring at weaning [ $F(1,38) = 4.004$ ;  $P = 0.053$ ; Table 1].

### 3.2. Corticosterone response

Corticosterone concentrations were similar in samples collected from control and CMS male offspring under restraint at 3 months of age. Thus no statistically significant differences or interactions related to gestational CMS exposure (data not shown).

### 3.3. Acoustic startle

At the age of 6 months, male rats were tested twice for ASR, with one week between tests. Animals habituated similarly to the initial five startle pulses, irrespective of prenatal CMS exposure and postnatal blood sampling. A statistically significant effect of Week [ $F(1,114) = 4.113$ ;  $P = 0.045$ ] indicated that overall, the basal startle response was slightly higher during the first compared to the second week of testing (Average startle during the first five initial startle pulses: Week 1:  $734 \pm 28$  (SEM); Week 2,  $651 \pm 27$  (SEM)). The average basal startle response during the initial 5, the middle 10, and the concluding 5 startle trials did not vary statistically significantly between groups (data not shown).

For PPI (Fig. 2a and b), overall loudness of prepulse trended positively with inhibition of the startle response, i.e. the louder the prepulse the higher the inhibition of the startle response. A main effect for Week was observed by reduced PPI for all four prepulses [PPI72:  $F(1,114) = 8.439$ ,  $P = 0.004$ ; PPI74:  $F(1,114) = 11.212$ ,  $P = 0.001$ ; PPI78:  $F(1,114) = 3.881$ ,  $P = 0.051$ ; PPI86:  $F(1,114) = 6.231$ ,  $P = 0.014$ ], at Week 1 as compared to Week 2, reflecting that the level of inhibition generally increased from Week 1 to Week 2 of testing (Fig. 2).

Association with prenatal CMS exposure and postnatal blood sampling under restraint was also indicated, as the interaction CMS × Blood sampling × Week was statistically significant for the two lowest levels of prepulse [PPI72:  $F(1,114) = 7.554$ ,  $P = 0.007$ ; PPI74:  $F(1,114) = 4.758$ ,  $P = 0.031$ ]. No other main effects or statistically significant interactions were present.

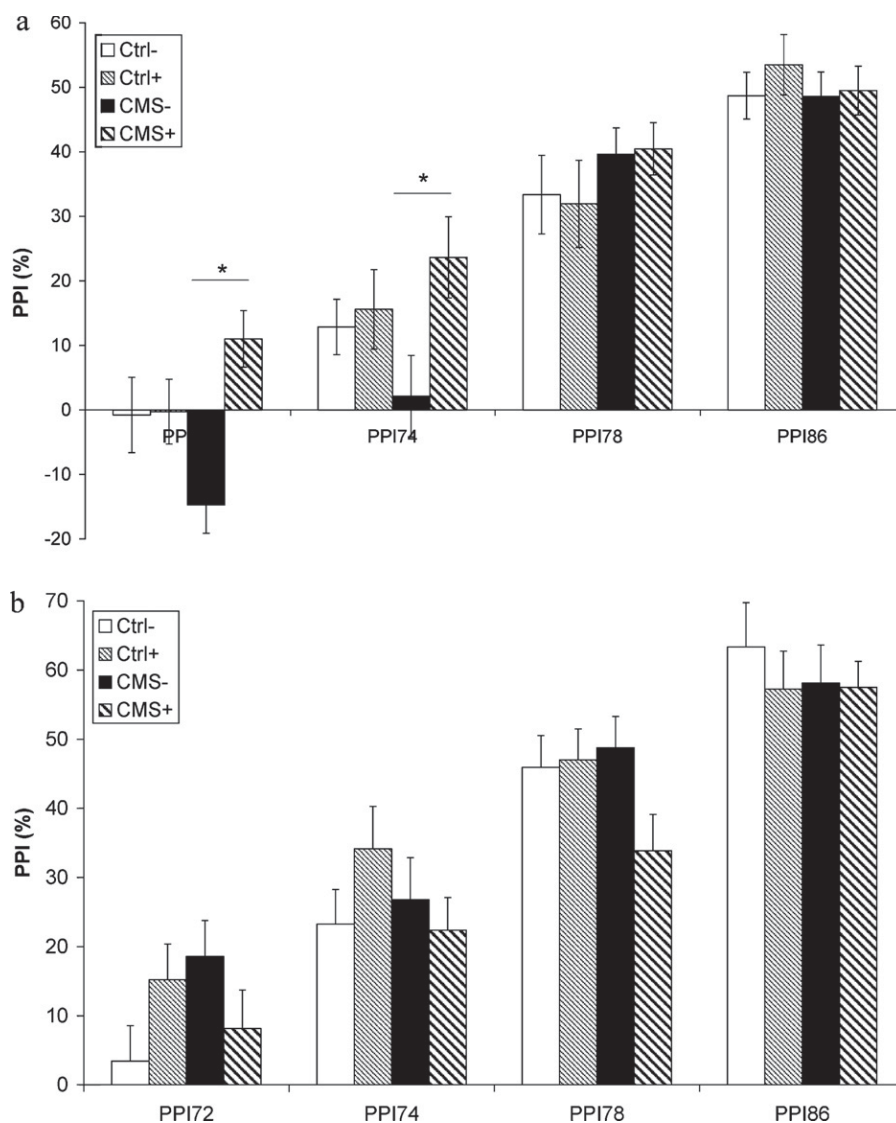
For PPI72, decomposition of the statistical interactions and separate analysis of data obtained during Week 1 indicated statistically significant effects of Blood sampling [ $F(1,57) = 4.348$ ;  $P = 0.042$ ] and of the interaction Blood sampling × CMS [ $F(1,57) = 4.015$ ;  $P = 0.050$ ]. Blood sampling was associated with significantly enhanced inhibition in blood sampled compared to non-sampled CMS offspring ( $P = 0.015$ ; Fisher's LSD), whereas no statistically significant difference was present for Blood sampling in control offspring (Fig. 2a).

For PPI74, Blood sampling showed statistically significant [ $F(1,57) = 4.552$ ;  $P = 0.037$ ] during the first week of testing. As for PPI72, Blood sampling was associated with significantly enhanced inhibition in blood sampled compared to non-sampled CMS offspring for PPI74 ( $P = 0.017$ ; Fisher's LSD). Again, no statistically significant difference was present for Blood sampling in control offspring (Fig. 2a).

Separate analysis of PPI72 and PPI74 data obtained during the second week of testing indicated no statistically significant main effects or interactions (Fig. 2b).

For PPI78 and PPI86, apart from week, statistical analysis indicated no main effects or statistically significant interactions.

Statistical analysis of LES data by 2 way ANOVA with Block as repeated measure revealed an effect of Block [ $F(4, 240) = 3.854$ ;



**Fig. 2.** Mean average PPI at the four levels of prepulses (72, 74, 78, and 86 dB(A)) in male offspring at Week 1 (a) and Week 2 (b) of testing during the dark phase at age 6 months. Overall statistical analysis showed statistically significant effect of Week for all four prepulses ( $0.01 < P < 0.052$ ) as well as of the interactions CMS  $\times$  Blood sampling  $\times$  Week for the two lowest levels of prepulse (PPI72:  $P = 0.007$ ; PPI74:  $P = 0.031$ ). Decomposition of these interactions indicated significant effects of Blood sampling ( $P = 0.042$ ) and of the interaction Blood sampling  $\times$  CMS ( $P = 0.050$ ) for PPI72, and of Blood sampling for PPI74 ( $P = 0.037$ ), during the first week of testing. Ctrl- = control – blood sampling; Ctrl+ = control + blood sampled at 3 months. CMS(-) = prenatal CMS–blood sampling. CMS+ = prenatal CMS + blood sampled at 3 months. Mean  $\pm$  SEM,  $n = 16$ . AVG: average amplitude of the startle response; and PPI: prepulse inhibition. \* $P < 0.05$ , Fisher's LSD.

$P = 0.005$ ], indicating that overall the basal startle response was increased in the light (blocks 5–8) compared to the startle response recorded in darkness (block 4; data not shown). No other factors (prenatal stress, blood sampling and interactions of factors) achieved statistical significance in this test.

#### 4. Discussion

The present study investigated whether stress during gestation would influence the startle phenotype of the male offspring. The main finding was a differential effect of postnatal blood sampling on PPI in male offspring from dams exposed to CMS during gestation. In these offspring the PPI depended on prior exposure to an episode of postnatal acute stress in the form of blood sampling under restraint. In contrast, PPI was unaffected by this postnatal experience in control male offspring. Control and CMS male offspring showed similar levels of basal startle, LES and corticosterone response to an acute postnatal stressor restraint.

For control offspring, PPI was almost indiscernible between Ctrl- and Ctrl+ at all four levels of prepulses. For prenatally stressed offspring, this only seemed the case for PPI78 and PPI86. For PPI72 and PPI74, blood-sampled CMS offspring displayed statistically significantly higher inhibition than non-sampled CMS offspring. The only groups that differed statistically significantly from each other were thus CMS- and CMS+ (i.e. non- and blood-sampled male offspring that underwent maternal CMS during gestation). The pattern of PPI in non-sampled CMS males bears some resemblance to that observed in a study of male rats that were subjected to maternal repeated variable stress in utero. Prepulse inhibition of the acoustic startle response was diminished across a range of prepulse intensities. Interestingly, startle testing was conducted in naïve offspring, i.e. startle testing was not preceded by any other behavioural testing or stressful events (Koenig et al., 2005). In contrast, PPI was increased in male offspring after maternal gestational restraint thrice daily or exposure to dexamethasone (Hauser et al., 2006; Kleinhaus et al., 2010; Lehmann et al., 2000). These latter findings are more in line with our observations in the CMS males that

had also been blood sampled. Furthermore, in the two studies with comparable periods of maternal exposure, startle testing was preceded by behavioural testing in the male offspring that displayed increased PPI. The contribution of these preceding and potential stressful experiences to the changes in PPI was not specifically investigated (Hauser et al., 2006; Lehmann et al., 2000). However, the prenatal stress effects on PPI was counteracted by maternal separation (6 h/day on PND 12, 14, 16, and 18, with pups in incubator at 25 °C) (Lehmann et al., 2000), emphasizing that PPI is indeed susceptible to modulation by postnatal events.

As reported previously, in female offspring from dams exposed to CMS during pregnancy PPI was decreased compared to controls. This was observed irrespective of postnatal blood sampling under restraint and at more or less all four levels of prepulses. Furthermore, postnatal blood sampling enhanced PPI in both control and CMS female offspring (Kjaer et al., 2010b). In male rats, postnatal blood sampling did not generally enhance PPI. This indicates a fundamental difference regarding the consequence of postnatal stress in male and female rats. Thus a single stressful life event induced long-term changes in female rats, irrespective of the prenatal environment. In male rats, a stressful life event solely imprinted those that had undergone fetal life in a stressful environment.

In our male CMS offspring, the direction of the PPI response therefore depended on the combination of pre- with postnatal stress. It could be inferred, that each of the CMS groups to some degree expressed parts of the female response pattern, i.e. somewhat low PPI after prenatal CMS but higher when combined with stressful blood sampling. Interestingly, male offspring from dams stressed during gestation have been reported to show some signs of feminization in several studies, e.g. by altered sexual behaviour, feminized juvenile play pattern and decreased size of the sexually dimorphic preoptic area in the brain (Holson et al., 1995; Reznikov and Nosenko, 1995; Ward and Stehm, 1991; Weinstock, 2007). No such endpoints were assessed in the present study, but feminization of males by prenatal stress is an important research area which warrants further work. Taken together, the same protocol of gestational CMS constitutionally change PPI in female offspring (Kjaer et al., 2010b), whereas in males, perturbation of the fetal environment left PPI sensitive to changes due to stressful events later in life.

LES is based on the natural aversion to brightly lit environments in nocturnal animals (e.g. rodents). The paradigm is especially interesting for research on emotional reactivity, because it is based on innate aversion. It does therefore not depend on cognitive functioning, a feature that seems to be altered in affective disorders and also in prenatally stressed animals (Bijlsma et al., 2010; Hougaard et al., 2005a). In previous studies from our laboratory, pregnant rats were stressed during gestation and their female offspring were tested for reactivity in the acoustic startle test. Maternal stress during fetal development increased the startle response in the offspring compared to controls. However, this has only been observed in the offspring that has been blood sampled under restraint months before startle testing (Hougaard et al., 2005a,b; Kjaer et al., 2010a). Experimental data indicate that the neural system mediating such long-duration aversive states probably involves the bed nucleus of the stria terminalis (BNST). The BNST is involved in sustained forms of potentiated startle that do not involve specific cues (Davis et al., 2010). LES is associated with a potential and temporally uncertain threat, leading to a sustained aversive state. This contrasts the short-duration aversive states due to fear of a specific threat, as in fear potentiated startle. Here the startle reaction is augmented due to expectation of a specific fearful stimulus, e.g. electrical shock (Grillon, 2008; Kjaer et al., 2010b). If the BNST was involved in the long-lasting effects observed in our previous studies, there was reason to hypothesize that also LES would be elevated in prenatally stressed animals. Interestingly, enhanced LES has been observed in

male offspring from dams stressed by cold during gestation (Tazumi et al., 2005). In the present study CMS male offspring displayed neither enhanced LES nor enhanced basal startle. This indicates lack of or little effect of the applied pre- and postnatal stress events on the proposed BNST-modulation of the startle response. The degree of prenatal stress experienced by our CMS males offers a tentative explanation for the altered PPI, but unaffected basal startle response compared to that of controls (Kjaer et al., 2010b). In the present study, stressed dams solely underwent CMS and control dams stayed undisturbed in their cages. This contrasts treatment in our previous studies, where both CMS and control dams were allocated to wire cages in a different animal room (Hougaard et al., 2005a) or received subcutaneous injections with vehicle (Hougaard et al., 2005b) on a daily basis. In the offspring from these additionally treated dams the basal startle response was increased whereas PPI went unaffected. The distinct startle phenotypes observed after different maternal treatments suggest that pure maternal CMS is indeed stressful for the offspring as indicated by the change in PPI. However, other modulations may come at play when CMS is combined with other potentially stressful components. If the level of stress exposure influences modulation of the ASR signalling pathway by higher order brain areas such as the BNST, it could be speculated that a higher stress level than pure CMS is needed for the animals to exhibit increased basal or LES (Kjaer et al., 2010b).

Prenatal stress has in several studies been associated with elevated or protracted corticosterone response to acute stressors (Glover et al., 2010; Weinstock, 2008). This endpoint showed resilience to prenatal stress in the present study, similarly to observations in female offspring in our previous studies (Hougaard et al., 2005a,b; Kjaer et al., 2010b). We previously proposed that timing of blood sampling during the 24 h cycle offered a potential explanation as to the lack of effects (Hougaard et al., 2005a), based on findings of increased basal levels of plasma corticosterone in prenatally stressed rats during the light but not the dark period (Koehl et al., 1997, 1999). However, changing monitoring of the hormonal stress response from the dark to the light period still left the corticosterone response to restraint unaffected in prenatally stressed female rats (Hougaard et al., 2005b). The intensity of gestational stress is a potential determinant of prenatal stress effects (Weinstock, 2008). In our previous studies in female offspring, exposure during gestation have consisted of pure CMS, CMS combined with either subcutaneous vehicle injections or daily allocation to wire cages, or injections with dexamethasone. In the latter study, dexamethasone clearly affected both dam and offspring, evidenced by lowered maternal gestational weight gain and birth weight (Kjaer et al., 2010b), but here again the corticosterone response to restraint was unaffected. Based on findings in female rats, the intensity of the gestational exposure does probably not explain lack of effect on corticosterone response to stressors in the present study. Factors apart from the intensity of prenatal manipulations may be at play. An educated guess points in the direction of the strain of rat (Skripuletz et al., 2010), i.e. the Wistar rats involved in our studies may be somehow resistant to hypothalamic pituitary adrenal changes by prenatal manipulations. Alternatively, an increase in the peak concentrations of corticosterone might only be detected if the applied postnatal acute stressor induces a sub maximal increase of plasma corticosterone in controls, i.e. lack of effect occurred due to a “ceiling effect” (Weinstock, 2008).

## 5. Conclusion

The present work showed that maternal gestational exposure to a relatively mild, variable paradigm of stressors affected the PPI response pattern in male rats. Prior exposure to a postnatal stressful event altered the direction of the PPI response in prena-

tally manipulated males. This differential PPI response resembled in part a PPI response pattern previously observed in female rats in our lab. In contrast, the PPI response in control males was unaffected by postnatal experiences. These data support the hypothesis that the maternal environment mediates of phenotypic differences in stress-related sensitivity on a long-term basis.

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### Conflicts of interest statement

None declared.

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