From the DEPARTMENT OF NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

EFFECTS OF EXCESS GLUCOCORTICOIDS ON NEURAL CELL FATE: LONG-LASTING CONSEQUENCES OF ADVERSE PRENATAL FACTORS

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Stockholm 2017

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Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7676-836-5

Effects of excess glucocorticoids on neural cell fate: long-lasting consequences of adverse prenatal factors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACADEMIC DISSERTATION

The thesis will be defended publicly in Petrén Lecture Hall,

Nobels väg 12B, Karolinska Institutet, Stockholm, Sweden

Monday, the 23th of October 2017, 09:30

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To my family

ABSTRACT

The developing nervous system is particularly vulnerable to high level of glucocorticoids (GCs). Excess GCs is often associated with decreased birth weight in relation to gestational age, and to increased risk of psychiatric disorders, like depression, later in life. Persistent deregulation of the hypothalamic-pituitary-adrenal axis drive on central and peripheral systems has been suggested to play a central role, however the link between developmental GCs exposure and late-onset depression is poorly understood. The aim of this thesis was to investigate and characterize behavioral alterations induced by developmental exposure to excess GCs in mice. Twelve month old mice (C57Bl/6) exposed to dexamethasone (DEX) (0.05 mg/Kg/day s.c.) from gestational day 14 until delivery displayed depression-like behavior resistant to treatment with the SSRI antidepressant fluoxetine (FLX), and decreased neurogenesis. These alterations, not detected at younger age, were associated with deregulation of genes promoting progenitor stem cells quiescence and proliferation, such as Cdkn1c. Neuronal stem cells exposed to DEX *in vitro* exhibited changes in methylation of promoter regions of the same class of genes, suggesting the involvement of epigenetic mechanisms. Depression-like behavior is often associated with altered hippocampal connectivity of granule neurons in the dentate gyrus. We investigated the morphology of adult-born hippocampal granule neurons and found remodeling in dendritic arborization accompanied by changes in the expression of TrkB, DISC1 and Reelin. In addition, DEX-exposed mice showed blunted circadian oscillations in corticosterone secretion, and down-regulation of glucocorticoid receptor expression in the hippocampus, which may explain the resistance to FLX treatment. We then tested the SNRI antidepressant desipramine (DMI), which reversed the depressed phenotype. Since modifications in corticosterone fluctuations are also associated with circadian rhythm alterations, we tested whether DEX might alter circadian rhythms. Twelve month old DEX-exposed mice showed abnormal circadian entrainment, which appeared to be more rigid and strongly dependent on photic drive. Interestingly, circadian alterations were displayed already at 6 months, long before the onset of depression-like behavior, and were reduced by chronic DMI administration. We assessed the function of the central clock and its drive on the hippocampus. The expression of arginine-vasopressin, the main output of the suprachiasmatic nucleus (SCN), was downregulated in DEX-exposed mice, suggesting a decoupling of the SCN control on the hippocampus. The core clock gene Bmal1 showed robust circadian fluctuations in the DEX-exposed mice SCN, while the oscillations were abolished in the hippocampus. The marked de-synchronization of Bmal1 across the SCN and hippocampus was restored by chronic treatment with DMI. Neither depression nor morphological and circadian alterations were developed in mice that received chronic treatment with DMI at 6 months, suggesting that the restoration of norepinephrine transmission might prevent the appearance of the phenotype. All together our results indicate that prenatal exposure to DEX triggers early changes in circadian rhythmicity, alterations in adult neurogenesis and neuronal plasticity preceding the onset of depression-like behavior.

LIST OF SCIENTIFIC PAPERS

I. Alterations in circadian entrainment precede the onset of depression-like behavior that does not respond to fluoxetine. Spulber S, **Conti M**, DuPont C, Raciti M, Bose R, Onishchenko N, Ceccatelli S. Translational Psychiatry, 2015, 5:e603

doi: 10.1038/tp.2015.94.

II. Depressive-like phenotype induced by prenatal dexamethasone in mice is reversed by desipramine. **Conti M**, Spulber S, Raciti M, Ceccatelli S. Neuropharmacology, 2017, 126:242-249

doi: 10.1016/j.neuropharm.2017.09.015.

III. Tet3 mediates stable glucocorticoid-induced alterations in DNA methylation and Dnmt3a/Dkk1 expression in neural progenitors. Bose R, Spulber S, Kilian P, Heldring N, Lönneberg P, Johnsson A, **Conti M**, Hermanson O, Ceccatelli S. Cell Death and Disease, 2015, 6:e1793

doi: 10.1038/cddis.2015.159.

IV. Desipramine restores circadian entrainment in mice exposed to dexamethasone in utero. **Conti M**, Spulber S, Raciti M, Ceccatelli S. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

11βHSD2	11beta-Hydroxidsteroid Dehydrogenase
5-HT	Serotonin
ACTH	Adreno Corticotropic Hormone
ADHD	Attention Deficit Hyperactivity Disorder
AVP	Arginine-Vasopressin
BDNF	Brain Derived Neurotrophic Factor
Bmal1	Aryl hydrocarbon receptor nuclear translocator-like protein1
CA(1;3)	Cornu Ammonis area
CAF	Commissural Associational Fibers
Cdkn1c	Cyclin-dependent kinase 1c
CREB	cAMP Response Element Binding
CRH	Corticotropic Releasing Hormone
DCX	Doublecortin
DD	Dark/Dark
DEX	Dexamethasone
DFA	Detrended Fluctuation Analysis
DG	Dentate Gyrus
DISC1	Disrupted In Schizophrenia 1
DMI	Desipramine
EC	Entorhinal Cortex
EdU	5-ethynyl-2´-deoxyuridine
ERK	Extracellular signal Regulated Kinase
FLX	Fluoxetine
FST	Forced Swim Test
Gap-43	Growth associated protein-43
GCs	Glucocorticoids
GD	Gestational Day
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenocortical
HSP	Heat Shock Protein
IUGR	Intrauterine Growth Retardation
IV	Intra-daily Variability
Klf15	Krüppel-like factor 15
LC	Locus Coeruleus
LD	Light/Dark
MAPK	Mitogen-Activating Protein Kinase
Mbd1	Methyl-CpG binding domain protein
MDD	Major Depressive Disorders
mo	months
NA	Nucleus Accumbens
NE	Norepinephrine
NSC	Neural Stem Cells
p16	tumor suppressor gene *p16 (CDKN2/MTS-1/INK4A)*

Per1	Periodic circadian protein homolog 1
PND	Post-Natal Day
PVN	Paraventricular Nucleus
Reln	Reelin
SAD	Seasonal Affective Disorders
SCN	Suprachiasmatic Nucleus
SGZ	Sub-Granular Zone
SNRI	Serotonin-Norepinephrine Reuptake Inhibitor
SSRI	Selective Serotonin Reuptake Inhibitor
TrkB	Tropomyosin-related kinase B
ZT	Zeitgeber Time

1 INTRODUCTION

1.1 BACKGROUND

Homeostasis and adaptation to environmental challenges are achieved through complex molecular mechanisms and drive behavior towards the best strategy for survival. *In utero* developmental programming assists early adaptation and prepares the embryo to adequately respond to the post-natal environment. Any excessive and continuous disturbance at different levels, particularly during critical stages of embryonal maturation, may lead to early life programming alterations and long term metabolic anomalies and psychiatric disorders in adult life, which might even be passed onto the next generation (Gluckman and Hanson, 2004). Over-exposure to glucocorticoids (GCs) has long been proposed as the most plausible link between adverse prenatal milieu and adult disorders (Reynolds, 2013).

High levels of cortisol in humans affects the biochemical milieu of the fetus and correlate with a greater incidence of prematurity and low birth weight (Field et al., 2009, 2006; Maina et al., 2008). Insufficient gestational growth is associated with high risk for hypertension, cardiovascular diseases, obesity and metabolic disorders, such as diabetes, as well as delayed neurological development, ADHD and depression in adulthood (Grissom and Reyes, 2013; Longo et al., 2012; Pesonen et al., 2009; Ra and Ja, 2014; Räikkönen et al., 2008; Strang-Karlsson et al., 2008). In pregnant women many factors contribute to increase the risk for GC over-exposure in the fetus: poor nutrition, stress and depression as well as maternal infection and inflammation, hypoxia and genetic deficiency of placental 11β-Hydroxysteroid Dehydrogenase (11βHSD2) all correlate with elevated cortisol which ultimately increase the risk for intrauterine growth retardation (IUGR) (Barker et al., 1993; Cottrell and Seckl, 2009; Martyn, 1994; Seckl, 2004).

Since the publication of a landmark trial by Liggins and Howie in 1972 (Liggins and Howie, 1972), synthetic GCs administration to pregnant women at risk of preterm delivery has become a standard procedure to significantly reduce infant mortality. The study showed that administration of synthetic GCs or glucocorticoid receptor (GR) agonists (such as dexamethasone (DEX) or betamethasone) in preterm labor could reduce the risk of infant respiratory distress syndrome. This led to a worldwide implementation of corticosteroid administration in cases of pregnancies with high risk of premature birth. Despite the synthetic corticosteroids' short term beneficial effects, epidemiological studies have shown that the long-term effects include persistently altered hypothalamic –pituitary-adrenocortical (HPA) axis response to stress, and possible adverse health effects in adulthood (Waffarn and Davis, 2012). Similarly, in animal models studies on synthetic GCs administration during pregnancy showed deregulation of the HPA axis accompanied by a GR gene expression decrease in the hippocampus (Levitt et al., 1996) altering the hippocampal drive on HPA axis activity (Sapolsky et al., 1984; Shoener et al., 2006), and resulting in HPA hypo-responsiveness (Sloboda et al., 2007). Prenatal exposure to excess GCs has been shown to also increase the susceptibility of neural cells to oxidative stress inducing modifications of the developmental

programming of the internal auditory system (Canlon et al., 2003) and altering the antioxidant levels and mitochondrial function in cerebellar granule cells (Ahlbom et al., 2000).

1.2 GLUCOCORTICOIDS

The end product of the HPA axis are the GCs, a class of steroid hormones that exert their effect through both mineralocorticoid receptors (MR) and GR. GCs reach their target cells and activate their intracellular receptors, which dimerize before binding to glucocorticoid or mineralocorticoid responsive elements in the nucleus to regulate (increasing or suppressing) transcription of specific genes. The biosynthesis and release of GCs is induced by the Adreno Corticotropic Hormone (ACTH) released in the blood from the anterior pituitary gland. ACTH release is triggered by the Corticotropin Releasing Hormone (CRH) produced by the parvocellular neurons in the paraventricular nucleus (PVN), transported to the external zone of the median eminence and then reaching the anterior pituitary through the portal vascular system. Circulating GCs mediate the stress response and provide negative feedback to the pituitary gland to inhibit ACTH release, as well as to the PVN to inhibit CRH release. As part of the regulatory feedback loop, hippocampal neurons, expressing receptors for GCs, send inhibitory input to suppress the hypothalamic release of CRH (Mastorakos and Ilias, 2003). Therefore, the hippocampus is both a target and a regulator of the brain's response to stress. Endogenous levels of GCs are tightly regulated not only by the HPA axis activity, but also depend on the activity of intracellular 11β-HSD enzymes, which convert active GCs into their inactive 11-keto metabolites (Sheppard, 2003).

During organogenesis, GCs induce cell proliferation, differentiation and maturation of several organs including kidney, cardiovascular system and gastrointestinal tract. Most importantly GCs promote lung maturation and surfactant production, which are essential for postnatal survival (Harris and Seckl, 2011; Khulan and Drake, 2012; Ward, 1984). GCs also promote brain development by initiating terminal maturation, remodeling of axons and dendrites, and affecting cell survival (Cameron and Gould, 1994; Meyer, 1983; Yehuda et al., 1989). Most notable is their role in the development and function of the limbic system and regulation of the HPA axis activity as early as 18 gestational weeks in humans.

GCs effects on developmental programming depend mostly on the time-frame of the exposure and can persist not only in adult life but can be passed onto the offspring, suggesting the involvement of epigenetic modifications (Drake et al., 2011, 2005). Indeed, alterations in gene expression consequent to stress exposure correlates with increased HPA axis responsivity, changes in CRH and GR expression associated with alterations in promoter methylation in animal models (Mueller and Bale, 2008).

1.3 NEUROGENIC THEORY OF DEPRESSION AND GLUCOCORTICOIDS IN THE HIPPOCAMPUS

The hippocampus is a highly plastic region of the mammal's brain that preserves neurogenic activity in adulthood and is integral to the regulation of stress responses (Anacker et al., 2013; McEwen, 2002). GC-sensitive hippocampal neurons are involved in terminating the

adrenocortical stress response (Sapolsky et al., 1984) but their beneficial effect on stress adaptation is compromised by excessive and prolonged exposure. Sub-chronic exposure to DEX inhibits adult hippocampal neurogenesis in rats (Kim et al., 2004) and promotes oligodendrogenesis (Chetty et al., 2014). The hippocampus is also vulnerable to developmental insults: administration of synthetic GCs in mice at post-natal day (PND) 6, affects the precursor neural stem cells niche size through adulthood (Kim et al., 2004; Ortega-Martínez and Trejo, 2014).

In addition, exposure to high levels of corticosterone affects the electrophysiological properties of immature neurons by reducing their activation in the ventral hippocampus in response to spatial and non-spatial memory tasks (Workman et al., 2015). de Kloet and colleagues reviewed a differential mechanism through which GCs directly affect hypothalamic and hippocampal regions: MR binding exerts a rapid effect on glutamatergic neurons while GR binding mediates a slower genomic response to stressors (de Kloet et al., 2008).

The differential binding to MR or GR has been proposed as a possible mechanism through which GCs regulates early and adult neurogenesis. MR activation increases hippocampal progenitor cell proliferation and promotes astrocytic differentiation; GR activation decreases proliferation and promotes neuronal differentiation, counteracting the MR-induced increase in astrogliogenesis. The proposed molecular mechanism is that GCs decrease neuronal differentiation by Notch/Hes signaling, FOXO3A signaling, TGFb-SMAD2/3-signaling and Hedgehog signaling (Anacker et al., 2013). In addition, GCs were reported to decrease proliferation of embryonic neural stem cells through Ubiquitin-mediated degradation of cyclin D1 *in vitro* (Sundberg et al., 2006), and Caveolin-1 seems to mediate the anti-proliferative effect of GCs (Peffer et al., 2014).

Developmental exposure to excess GCs has been associated with higher risk for late-onset psychiatric disorders, and with depression in particular (Harris and Seckl, 2011; Räikkönen et al., 2008). According to the monoamine hypothesis, depression is the result of serotonin (5-HT), norepinephrine (NE), and dopamine (DA) neurotransmission imbalance in specific brain regions (Hamon and Blier, 2013). The neurogenic theory of depressive disorders emphasizes the link between hippocampal neurogenesis and depression: decreased neurogenesis following chronic stress can reduce the inhibitory activity of the hippocampus on the HPA axis (feedback loop). The suggested consequences are HPA axis hyperactivity, increased blood levels of stress hormones, and hippocampal damage, all common features of major depressive disorders (MDD). Nevertheless, the relationship between structural hippocampal changes and depression is still unclear (Dranovsky and Hen, 2006). The monoamine hypothesis and neurogenic theories of depression converge in that 5-HT depletion inhibits adult neurogenesis (Brezun and Daszuta, 1999), while chronic, but not acute antidepressant treatment promotes neurogenesis in the sub granular zone (SGZ) of dentate gyrus (DG) (Duman et al., 2001; Malberg et al., 2000; Wang et al., 2008). In addition GCs regulates the progenitor cells sensitivity to 5-HT receptor activation in the DG (Huang

and Herbert, 2005) while the HPA axis can influence the sensitivity of the 5-HT system by affecting the binding of 5-HT to its transporter (Figlewicz, 1999). The majority of known antidepressants, such as serotonin and/or noradrenaline reuptake inhibitors, serotonin 5-HT$_{1A}$ receptor agonists, and the melatonin receptor agonist / serotonin 5-HT$_{2B/C}$ receptor antagonist agomelatine, increase proliferation and maturation and survival of newborn hippocampal cells in rodents (Duman et al., 2001, 1999; Malberg and Duman, 2003; Wang et al., 2008). The effect of 5-HT on neuron proliferation depends on the recruitment of different types of 5-HT receptors (Banasr et al., 2004; Jacobs et al., 2000; Santarelli et al., 2003) with a preferential involvement of 5-HT1A receptors. A 3-week treatment with the SSRI fluoxetine (FLX) in rodents increases cell proliferation in the DG by 70% (Duman et al., 2001), but this effect is not present in 5-HT1A receptor knock- out mice (Santarelli et al., 2003) or in animal models with ablation of hippocampal neurogenic niche by irradiation (Czéh et al., 2001; Santarelli et al., 2003).Therefore it has been proposed that the antidepressant effects may be mediated by hippocampal cell proliferation counteracting the reduced neurogenesis observed in animal models of depression and possibly in human patients with MDD (Masi and Brovedani, 2011).

1.3.1 Granule cells morphology in the adult hippocampus: implication for depression

The mood-improving action of antidepressants do not solely depend on neurogenesis restoration but it is also associated with neuronal remodeling (Bessa et al., 2009; David et al., 2009; Miyamoto et al., 2011; Seo et al., 2014) through monoamine direct actions (Rojas et al., 2014; Yan et al., 1997), and up-regulation of genes involved in neuronal proliferation, differentiation, and plasticity (Förster et al., 2006; Llorens-Martín et al., 2016). FLX and desipramine (DMI), for instance, have been reported to restore not only neurogenesis (Chen et al., 2006; Guirado et al., 2012), but also the complexity of dendritic arborization and the density of dendritic spines in the hippocampus (Laifenfeld et al., 2002; Norrholm and Ouimet, 2000). Changes in hippocampal function and in the morphology of hippocampal neurons have been described in depressed patients as well as in animal models of depression (Campbell and MacQueen, 2004; Kulkarni and Firestein, 2012; Magariños et al., 1996; Miller and Jacobs, 1984; Watanabe et al., 1992).

The morphology of granule neurons plays a key role in the physiological function of the hippocampus: dendrites from the hippocampal granule cells spread throughout the inner and outer molecular layer, where commissural associational fibers (CAF) from contralateral or ipsilateral granule cells and entorhinal cortex (EC) projection, respectively, establish synaptic connections. The reduction in dendritic complexity and synaptic spine density in the molecular layer may reflect a disconnection of the EC inputs to the hippocampus with consequent impairment of hippocampal function (Llorens-Martín et al., 2016).

1.3.2 Disruption of circadian rhythms and neuropsychiatric disorders

The circadian clock is an internal oscillator identified in all living organisms, which allows the synchronization of biological function to the light-dark cycle (Ko and Takahashi, 2006). In mammals, including humans, this function is performed by a population of neurons located in the SCN. The SCN consists of several neuronal populations that display prominent cyclic fluctuations in firing patterns. The activity of SCN networks synchronizes the circadian fluctuations in physiological functions, including hormonal and autonomic regulation of metabolism with the dark-light cycle (see (Albrecht and Oster, 2001; Kiessling et al., 2010; Leliavski et al., 2014) (Fig. 1-1). The most important signal that is able to reset the circadian clock and synchronize it with an externally imposed rhythm is light. Thus, in addition to merely keeping the pace for spontaneous activity, the SCN function also has a degree of plasticity/adaptability. The regular distribution of activity and resting/sleep periods is an example of output of the circadian clock (Hu et al., 2009).

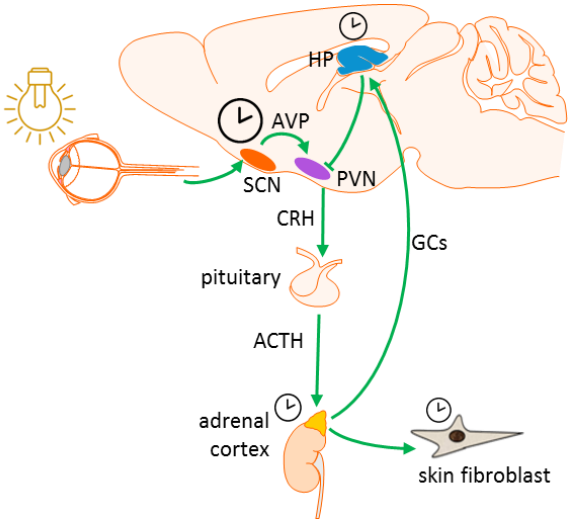

Figure 1-1 Schematic representation of the timing system. The central clock, located in the SCN, is entrained by environmental light via the retino-hypothalamic tract. It controls the activity of the HPA axis via AVP release to the PVN. Circulating GCs entrain peripheral oscillators (e.g. skin fibroblasts) as well as slave oscillators located in the brain. The hippocampus contributes to the feedback loop by inhibiting the release of CRH from the PVN.

Arginine-vasopressin (AVP) is the main output from SCN (Kalsbeek et al., 2010) and plays a critical role in driving slave oscillators (*e.g.* hippocampus) through HPA axis rhythmic activity (Buijs et al., 1999). Within the SCN, AVP signaling is critical for the synchronization of neuronal firing patterns, and decreased AVP signaling in the SCN renders the self-sustained oscillation more sensitive to re-entrainment (Yamaguchi et al., 2013). The SCN expresses alpha-adrenoceptors (Morien et al., 1999) and receives noradrenergic innervation from locus coeruleus (LC) (Cagampang et al., 1994). Although the role of NE in regulating the SCN is not clear it has been proposed that NE modulates the expression of AVP in SCN in a circadian fashion and may account for the mechanism of action of medications (*e.g.* SNRI) for the treatment of psychiatric disorders often associated with circadian asynchrony (O'Keeffe et al., 2012; Someren, 2010; Wirz-justice, 2009). The relationship between circadian rhythm disruption as the result of variation in environmental cues (light/dark ratio and light pollution) and occurrence of depression has been reviewed by Salgado-Delgado and colleagues (Salgado-Delgado et al., 2011). The author suggests that the lack of synchronicity between internal rhythmicity in GCs and neurotransmitters concentration, and exposure to

time indicators such as light, feeding schedules and physical activity play a key role in etiopathogenesis of depression (Salgado-Delgado et al., 2011). Similarly, changes in molecular rhythms in SCN and in Nucleus Accumbens (NA) have been reported subsequent to induction of depression in animal models (Logan et al., 2015). The connection between circadian rhythms and MDD is supported by the following lines of evidence:

1) mutations in clock genes are associated with depression (MDD, seasonal affective disorder (SAD), as well as sporadic depressive episodes) (Albrecht, 2013; Lavebratt et al., 2010; Partonen et al., 2007)
2) interfering with normal circadian rhythms (*e.g.*, shiftwork) increases the risk of developing MDD or precipitates the recurrence of MDD episodes (Scott et al., 1997)
3) SAD occurs during winter, and is triggered by short light phase during winter months. In animal models, exposure to either continuous darkness or continuous light for extended periods of time leads to depression-like behavior (Tapia-Osorio et al., 2013)
4) therapeutic approaches aimed at restoring/resetting/regulating the circadian rhythms are most often effective in controlling mood. Moreover, treatment with melatonin (a hormone secreted by the pineal gland only during the dark phase, and controlled by direct input from the retina), or agomelatine (a melatonin receptor agonist with established antidepressant effects) are effective mood stabilizers (Mairesse et al., 2013; Marrocco et al., 2014)

The analysis of single genes variants in animal models of depression and in postmortem human samples with clinical history of MDD has so far provided information about very specific and restricted landmarks of circadian rhythm alterations and concomitant depression: variants of single clock genes in animal models has proven to correlate with abnormal sleep behavior but the normal rhythm appears to be disrupted only in response to variation in environmental cues suggesting that a broader family of gene contributes to specific phenotypes (Jones et al., 2013). Genome-wide association studies for sleeping disorders have so far shown a non-significant correlation between clock genes variants and sleeping disorders indicating that a functional genomic (transcriptomics) approach might prove to be more reliable. In fact it was shown that circadian patterns in the transcriptome of healthy donors are in-phase across six regions analyzed: SCN, dorsolateral prefrontal cortex, anterior cingulate cortex, hippocampus, amygdala, NA, and cerebellum. The patterns involve several hundred transcripts, led by the best-known clock genes such as BMAL1. Authors report that gene expression rhythms are intrinsically stable and inert to sudden changes in environmental cues outside of the SCN (Li, 2014).

At a subcellular level, the core of the molecular clock consists of a network of transcription factors, referred to as the clock genes, engaged in interlocking feedback loops (Ko and Takahashi, 2006). The cyclic function of the clock maintains a large degree of adaptability by integrating information on metabolic status and level of activity with environmental cues (*e.g.* ambient light intensity) in order to stabilize the 24 h periodicity. Interestingly, in the SCN and

hippocampus, alterations in synchronous neural activation due to variation in light exposure change the DNA methylation patterns (Azzi et al., 2014; Molyneux et al., 2008).

1.3.3 Glucocorticoids and circadian rhythms

GCs in adults regulate daily events and coordinate sleep activity by their intermittent secretion from the adrenal glands through the HPA axis activity, but can readily rise in response to stress. At a basal level, GCs follow ultradian and circadian rhythms with peaks occurring before the onset of the active phase in mammals (de Kloet et al., 2008). The ultradian and circadian modulation of GCs secretion is finely tuned by differential binding to MR or GR in target cells controlling basal homeostatic functions. In addition, the response of the adrenal cortex to ACTH signaling is gated by an intrinsic mechanism that depends on cyclic expression of clock genes.

At cellular level, the timing of GCs action is determined by GRs availability and heat shock protein (HSP) regulation: GCs bind to GR-HSP complexes and then translocate to the nucleus and interact with glucocorticoid response elements (GREs) on their target genes. The cycle is completed when GRs lose their ligand in the nucleus and are recycled through nuclear HSPs in the cytoplasm, ready for new binding to HSP and GCs. Interestingly the GR-GRE binding and release cycles is subordinated to HSP-mediated cycle (de Kloet et al., 2008; Dickmeis et al., 2013). It should be noted that these events occurring in a time-scale of minutes are superimposed onto a family of genes with intrinsic cyclic activity like clock genes: the CLOCK/BMAL1 target gene Per1 contains a GRE element in its promoter that is rhythmically bound and regulated by GCs in ultradian pulses both in cultured cells and in the hippocampus (Conway-Campbell et al., 2010; Stavreva et al., 2009). Moreover CLOCK regulates in a negative-feedback loop the GCs activity inhibiting the GR by acetylation and CRY activation (Lamia et al., 2011; Nader et al., 2009). Thus, it is proposed that there is cross-talk between the circadian clock gene machinery and GR signaling at both the transcriptional-regulation level, and the direct protein interaction level.

Clock genes are responsible for a transcriptional and translational auto-regulatory loop of molecular oscillation not only in the SCN, but also in peripheral tissues (Ko and Takahashi, 2006). They are expressed also in adult neuronal stem cells and are correlated with adult neurogenesis (Matsumoto et al., 2011) in relation to dark/light cycles (Guzman-Marin et al., 2007). Studies on neurosphere derived from murine neural stem/progenitor cells have revealed that Clock and Bmal1 are required for neurosphere formation, proliferation and cell survival. Furthermore, neurospheres display circadian rhythm in mPer1 expression, which regulates neurogenic transcription factors such as NeuroD1 (Kimiwada et al., 2009). Commitment to a neuronal fate is dependent on Bmal1 expression (Malik et al., 2015). Bouchard-Cannon and colleagues have shown that the molecular clock controls cell proliferation through Bmal1 and Per1 regulation of the cell cycle entry and exit and by mean of a cyclin dependent kinase inhibition (Bouchard-Cannon et al., 2013). More recently Watanabe and colleagues have proposed a specific mechanism for regulation of the timing of neuronal differentiation through an Hbp1 mediated control of cell cycle progression during

cortical development (Watanabe et al., 2015). Interestingly, in an *in vivo* analysis of adult progenitor cell differentiation, circadian variations seem to correlate more often with gliogenesis rather than with neurogenesis (Kochman et al., 2006). These observations underline the important influence of the clock genes on neurogenesis regulation.

In the vast spectrum of symptoms of MDD and Bipolar Disorders (BD), sleep disturbance and diurnal mood variation are among the most common (Hall et al., 1964; Riemann et al., 2002), suggesting the possibility of clock dysfunction in relation to GCs fluctuations.

Despite the abundant knowledge achieved during the last few decades on the field, the specific mechanisms through which developmental GCs exert their long lasting pleiotropic effects in mammals remain unclear. The impact of GCs on hippocampal neurogenesis and neuronal morphology, in relation to depression-like behavior, and circadian alterations require further investigation. The evaluation of appropriate pharmcological approaches to the complex and long-lasting pathologic condition resulting from GCs *in utero* exposure needs to take in account possible mechanisms not yet considered as plausible candidates.

2 AIMS

The general aim of this thesis was to explore the impact of deranged prenatal hormonal milieu induced by excess GCs in mice.

The specific objectives of the study were:

- **A.** To investigate the effect of prenatal DEX exposure in relation to late onset depression-like behavior and possible causal link with other behavioral changes, namely circadian rhythm alterations;
- **B.** To assess the impact of DEX on hippocampal neurogenesis and maturation of adult-born neurons in relation to behavioral alterations;
- **C.** To evaluate different classes of antidepressants and their possible regulatory mechanism on neurotransmission, neurogenesis, and neuronal morphology.
- **D.** To explore possible molecular mechanisms underpinning the phenotypical alterations consequent to developmental exposure to DEX.

3 MATERIAL AND METHODS

The purpose of the following section is to offer an overview on the experimental design and methodological consideration that arose in the planning and realization of the projects composing the thesis. The reader will find detailed description of each experimental procedure in each constituting paper.

3.1 ANIMALS AND EXPOSURE

All experiments were performed in agreement with the European and Swedish national regulation following approval by the local Animal Ethics Committee (Stockholms Norra djurförsöksetiska nämnd).

The exogenous administration of GC during early developmental stages leads to lower birth-weight, and in utero growth restriction/retardation (IUGR). There is increasing evidence that IUGR increases the risk of psychiatric disorders later in life (Anacker, 2014; Räikkönen et al., 2008; Strang-Karlsson et al., 2008). An adverse perinatal environment appears to have detrimental effects not only on HPA axis regulation, but also on the programming of the SCN, ensuing alterations in circadian rhythms often associated with depression in humans (reviewed in (Kennaway, 2002)). GCs-induced IUGR models are highly relevant, since GCs administration to women threatened by premature labor is widely used in clinical practice as commented in the introduction (Haugaard and Bauer, 2001). IUGR provides a model for metabolic stress that leads to acquired allostatic load as a chronic state imbalance in response to stress (maladaptation), and it is strongly related to behavioral alterations later in life (Anacker, 2014).

In this thesis we used a model of GCs developmental exposure in C57Bl/6 mice which leads to fetal growth retardation, and postnatally to lower bodyweight until the age of 4 weeks. The choice of mice versus rats was originally made in the perspective of the possible implementation of transgenic animals, mostly common in mice. We chose C57BL/6J mice because no particularly high emotionality is described for this strain and behavioral alteration should be displayed solely as the result of prenatal DEX exposure. Timed-pregnant dams (Charles River, Germany) were injected subcutaneously with 0.05 mg/k/day DEX (Sigma-Aldrich, Sweden) dissolved in sterile saline or equivalent volume of vehicle (10 mL/kg b.w. physiological saline) from gestational day (GD) 14 (the day the postcoital plug was considered GD 0) until delivery (recorded as PND 0). This dose was chosen to induce a moderate fetal growth retardation without affecting litter size, gestational length or maternal behavior (as assessed by pup retrieval test described below)(Celsi et al., 1998). A preliminary battery of behavioral tests showed alterations only in DEX-exposed males therefore we used only male offspring for subsequent studies.

The procedure for offspring handling is described in details in each paper.

The litters were culled to 4 pups per litter at PND 3 and the weight was monitored at PND 3, 7, 14 and 21. At PND 21 the mice were weaned in groups of 4-5 mice per cage and

tagged with subcutaneous radio frequency identification (RFID) transponders under brief isoflurane anesthesia for unambiguous identification of the pups throughout the experiments. The transponders were also used for monitoring the animal activity in their homecage environment. After implantation, the pups were redistributed to new cages so that each cage would house a maximum of five mice originating from different litters and the distribution was maintained throughout the study. Mice were kept in an animal facility under 12:12-h light–dark (LD) cycle (light intensity 50 lx; light on at 0600 hours) at constant temperature (22±1 °C) and humidity (50±5%).

3.2 BEHAVIORAL TESTS

The choice of behavioral test in the emotional domain was intended to minimize the exposure to stressful events related to unnecessary handling. The number of animals for the planned tests were decided based on the test with known higher variance (higher number required), and on meta-analysis on our strain, in order to establish a good compromise between a reasonable number of animals, high power and ethical considerations for animal use. In a preliminary set of experiments FST revealed an 80% power with 8 animals per group given the fairly low variance in our model. The assessment of spontaneous activity in the homecage using the TraffiCage™ system is particularly relevant for our approach and provides a valuable tool for unmasking specific anomalies related to prenatal DEX exposure.

3.2.1 Pup retrieval test

Since prenatal DEX exposure is achieved by DEX s.c. injection in E14 pregnant dams, and to rule out any possible confounding independent variables such as maternal behavior as effect of DEX treatment, we evaluated maternal care by means of a pup retrieval test. The test was adapted from (Umemura et al., 2015): briefly, pups at PND 3 were separated from their mothers for 10 min and only four male pups were returned in the respective mother's homecage, and placed at the more distant corners from the nest. The behavior was recorded for 10min using a video camera and analyzed offline by one investigator who was blind to the treatment conditions. Before the beginning of the recording session the quality of nest building was assessed by scoring from 1 to 4 (1 corresponded to disorganized nest structure or inconsistent use of nesting material; 4 to regular nest building and constant care of nest consistency). Three maternal behaviors were measured during the video analysis: the latency to first retrieval; time for each pup retrieval to the nest; the total time spent in the nest for maternal care; the time spent apart from the nest which was considered as neglecting behavior.

3.2.2 Forced Swim Test (FST)

The assessment of depression-like behavior was conducted with the FST at different ages and before and after each antidepressant treatment. Also known as Porsolt's test (named after the scientist who first proposed it in 1977) the FST is based on the principle that an animal exposed to an aversive stimulus will attempt to escape until it will stop and become

immobile. The duration of immobility is used to score depression-like behavior. The animals are individually placed for 6 min in glass cylinders filled with water (23°C), and test sessions are videotaped and analyzed offline. We considered immobility as passive floating for at least 2 seconds. In virtue of its high predictivity, reliability and reproducibility, FST is probably the most frequently use paradigm for the measurement of depression-like behavior and for testing antidepressants in rodents. The different behaviors displayed in response to aversive stimuli (swimming, climbing, struggling, and floating) have been proven to be associated with specific neurotransmitter activity, and hence predictive for various classes of antidepressant responsiveness (Cryan and Mombereau, 2004). Other paradigms for assessment of depression are known to either produce similar results (*e.g.* tail suspension test) or require shock presentation or long-term manipulation and training (*e.g.* learned helplessness, novelty-induced hypophagia test). We have confirmed the depression-like behavior in our model by tail suspension test. In later replicates, we observed a very consistent pattern of alterations and did not use the tail suspension test in order to minimize the animal stress and discomfort.

3.2.3 Spontaneous Activity

The monitoring of spontaneous activity is a non-invasive powerful tool to unmask behavioral alterations related to pathological conditions and more subtle mood disorders (Dawkins, 2006; Weary et al., 2014). The analysis of spontaneous behavior, which takes advantage of a more ethologically valid environment (Peters et al., 2015), has a prominent role in this thesis especially in relation to the effects of changes in light/dark conditions. Rodents are nocturnal mammals and display most of the exploratory activity during the dark phase in 24-h cycles with high degree of adaptability to changing in light-dark conditions. Continuous monitoring in a social and stimulating homecage environment with a minimal (although inevitable) interaction with the experimenter, can: (1) provide important information about undisturbed animals' activity level and distribution during active (dark) and inactive (light) phases; (2) reduce the introduction of confounding factors. We recorded the spontaneous activity of group-housed, freely moving mice using the TraffiCage™ system (NewBehavior, Zürich, Switzerland) which consists of an array of antennas embedded in a plate positioned under the homecage. The antennas can read the unique identification numbers stored in transponders implanted subcutaneously (RFID) and provide an approximate location of each animal with a time resolution of 20 ms. The system is able to detect the movement of each animal from one region of the cage covered by one antenna to a region covered by a different antenna and estimate the time spent in each region. A 'visit' is defined as the time interval during which an animal is detected constantly by the same antenna, and is used as activity count. The time series of visits are exported as ASCII files and analyzed using custom algorithm implementations in Matlab™ (The MathWorks, Natick, MA, USA). To minimize the confounding effects of novelty, we derived the baseline measurements based on three LD cycles after an acclimation period of at least three LD cycles. The circadian zeitgeber (German, 'time giver') time (ZT) 0 corresponds by convention to the onset of the light phase.

For the analysis of the active phase in relation to the LD cycle the time series of visits were binned in 5 min non-overlapping epochs, then smoothed with a weighted average using a sliding Gauss window (2 h width). The epochs with activity above the individual's detrended average were considered "active epochs". The active phase was defined as a sequence of active epochs either contiguous or separated by gaps no larger than 1h. The onset and the offset of the active phase were defined as the ZT corresponding to the beginning and the end of the active phase, respectively. The duration of the active phase was calculated as the time span between the onset and the offset of the active phase during one LD cycle. For steady entrainment conditions, the analysis of active phase is based on three consecutive LD cycles. We considered spontaneous activity as entrained to the light-dark cycle if the onset of the active phase occurred within 1 h from the onset of the dark phase. The individual delay in re-entrainment was estimated as the first day spontaneous activity was entrained to the shifted light-dark cycle.

To analyze the synchronization of spontaneous activity with the light-dark cycle, we recorded spontaneous activity in several conditions. A period of two weeks in constant darkness (DD; free- running period) was used to measure the spontaneous activity in free-running conditions, while the ability to re-entrain to normal conditions was tested by resuming the LD cycle. We also challenged the mice to re-entrain to the LD cycle by abruptly shifting the light offset. This paradigm is particularly suited to address the complex interactions between the internal clock and peripheral oscillators, because it allows the dissection of behavior features either SCN-specific, or pertaining to entrainment of activity as peripheral oscillator (*i.e.* independent from SCN).

The analysis of circadian rhythmicity consisted of rhythmometry by means of cosinor analysis (Nelson et al., 1979; Refinetti et al., 2007). The period of spontaneous activity was estimated as the highest peak in the $\chi 2$-periodogram (Sokolove and Bushell, 1978) between 20 and 25 h, with a 5-min resolution.

We analyzed the variability of spontaneous activity by estimating the intra-daily variability (IV), and by means of detrended fluctuation analysis (DFA). Both methods describe the patterns of fluctuations in spontaneous activity without making assumptions about the periodic nature of the fluctuations, or about the shape of the cyclic fluctuations (*e.g.* sinusoidal or square waves). IV describes the instantaneous variability in activity, while fractal analysis describes the complexity of series of fluctuations over time.

IV characterizes the patterns of activity within a 24 h cycle from the point of view of variations between consecutive time-bins. It was calculated as the ratio between the average squared differences between consecutive time-bins (pooled activity over 5 min in our case) and the global variance over the entire day (Gonçalves et al., 2015, 2014). It typically has values between 0 and 2; low values indicate smooth fluctuations between active and inactive episodes, while high values indicate fragmented activity patterns (Gonçalves et al., 2015, 2014).

Fractal analysis had been proposed as an analytical tool to distinguish between animals operating in normal versus pathological states (see (Macintosh et al., 2011)). DFA measures long term autocorrelation in data derived from sequential patterns of activity. The method is based on linear regression analysis of the residual variance of the time series against the timescale used for detrending on double-logarithmic plots. The intervals for detrending ranged from 20 min to 21.3 h in exponential increments. The correlation coefficient of a linear regression in double-logarithmic plot translates into a scaling exponent, and describes the long-term autocorrelation patterns embedded in the time series (Peng et al., 1995). Scaling exponent of 0.5 represents a non-correlated, random sequence (white noise), while scaling exponent $\neq 0.5$ indicates that the sequence displays long-range autocorrelation, *i.e.* the durations of a bout of activity depend on the duration of other bouts. If the scaling exponent has a value < 0.5 long bouts are more likely to be followed by short bouts and vice versa. If the scaling exponent is > 0.5, the sequence is persistent, long bouts are more likely to be followed by long bouts. The scaling exponent in young, healthy rodents and humans is around 0.8 (Hu et al., 2012, 2007). Values approaching 1 can be interpreted as reductions in complexity with strong underlying regularity (Peng et al., 1995), and are a hallmark of disease (Macintosh et al., 2011).

3.2.4 Analysis of synchronization of peripheral oscillators with the SCN

The central clock located in the SCN synchronizes all biological processes and behaviors to match peripheral oscillators with the LD cycle. While the main purpose is to maintain the overall synchronization, the SCN function must also have a degree of plasticity to allow the organism to adapt to changes. An abrupt change in the LD cycle induces a transient desynchronization of all oscillators with complex, but reversible biological disturbances as observed in the jet-lag syndrome. Therefore the integrity of SCN functions and its control on peripheral oscillators guarantees the ability to re-entrain at appropriate time. Mice were killed at the beginning of the subjective night (active phase) at different time points (N=2 / time point /group) every 1.5 h by an overdose of anesthetic (sodium pentobarbital, 150 mg kg^{-1}), and tissue samples from the ear were harvested before perfusing transcardially with ice-cold buffered saline followed by 4% paraformaldehyde. Brains were immediately dissected and RNA was extracted from different regions as described in the dedicated section.

The mRNA expression of Bmal1 was first normalized to have 0 average and unit variance relative to the group and brain region (standard deviation scores). The state portraits were obtained by plotting the relative Bmal1 expression for each animal in the SCN and the hippocampus on x and y axes, respectively (one animal – one datapoint, connected by a line to illustrate the sequence of times of death). Since the diurnal fluctuations in Bmal1 expression can be approximated by a sinusoidal curve, and the sinusoids for any two brain regions are in a constant phase relationship, the resulting plot should be a segment of ellipse. We approached the analysis of the state portraits as Lissajous figures, where the phase difference between the two sinusoids determines the orientation of the axis of the ellipse, and the direction of progression (Fig. 3-1). Thus, the state portraits provide an

illustration of the phase relationship, or lack thereof, between the two brain regions analyzed.

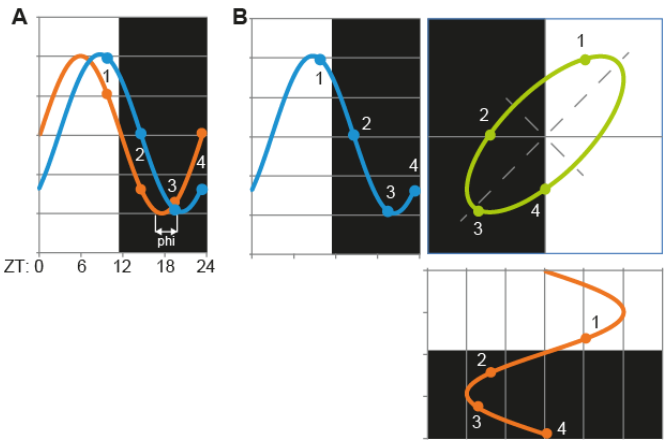

Figure 3-1 Lissajous figures result from the composition of two phase-locked sinusoids. (A) Depiction of variations over time using a common timeline (phi = phase difference). Four samples with discrete distribution over time are shown as 1-4. (B) The state portrait is constructed by plotting the sampled values on orthogonal axes. The orthogonal projection of the sinusoids describe an ellipse. The orientation of the major axis and the direction of rotation (order of sampling points) depend on phi. Note that time of sampling is not included in the state portrait as an independent axis.

3.2.5 Analysis of Bmal1 expression in skin fibroblasts

Skin fibroblasts express functional molecular clock machinery (Nagoshi et al., 2004; Welsh et al., 2004), and the circadian oscillations in clock gene expression maintain to a large extent the features of circadian rhythms in the central clock (Brown et al., 2008; Pagani et al., 2010). The molecular clock machinery in the fibroblasts acts as a peripheral oscillator and is subject to entrainment by the central oscillator (located in the SCN) (Brown et al., 2008), similar to spontaneous activity (Yamazaki et al., 2000). The possibility to synchronize self-sustained oscillations, and to reset the phase is preserved in cultured fibroblasts (Bamne et al., 2013; Welsh et al., 2004). We investigated the expression of clock genes in fibroblast isolated from all groups at the age of 6 mo. Skin fibroblasts cultures from the tissue samples were prepared as described in detail in Paper I. After synchronization (by exposing the fibroblasts to 1μM DEX), the cells were harvested at different time points, mRNA was extracted and the relative expression of Bmal1 was assessed by quantitative PCR with Gapdh as the housekeeping gene (see also Supplementary Materials in Paper I). The oscillations in Bmal1 mRNA expression were analyzed by cosinor rhythmometry (Nelson et al., 1979; Refinetti et al., 2007).

3.3 CORTICOSTERONE METABOLITES IN FECES

We investigated the diurnal rhythm of GCs secretion at 12 mo by collecting spontaneous fecal boli between ZT1-2 and ZT12-14 (*i.e.* immediately after the transition between the light and dark phases). The feces were collected in sterile Eppendorf tubes and stored at −80°C until further processing. Samples from each mouse (N=8–10 per group) were collected on two occasions (7-day interval between samplings). The concentration of corticosterone metabolites in dry fecal extracts was measured by enzyme immunoassay according to the manufacturer's instructions (DetectX, Arbor Assays, Ann Arbor, MA, USA).

3.4 RETROVIRUS MEDIATED LABELING OF NEWBORN NEURONS IN THE HIPPOCAMPUS

There are two regions in the adult brain where the neurogenic potential is retained: the subventricular zone (SVZ) (lining the medial aspect of the basal ganglia), and the subgranular zone (SGZ) of the DG. While the functional role of adult neurogenesis in humans is still debated, experimental evidence clearly links alterations in learning and mood with alterations in hippocampal neurogenesis. In rodents, the progenitor cells in the hippocampal neurogenic niche are generated at specific developmental age (GD14) and are preserved throughout life in a quiescent state (Clarke and Van Der Kooy, 2011). They respond to environmental cues and endogenous programming to provide functional, adult born neurons. One of the main objectives of this thesis was to characterize the morphological alteration in the mature granule cells of the hippocampus of DEX-exposed mice. In our model, the exposure to DEX in utero starts at GD14, *i.e.* the time the progenitor cells in the neurogenic niche are generated. The importance of neuronal integrity resides in the functional impact on the brain regions they innervate.

From the first use of the Golgi staining from Santiago Ramón y Cajal, alternative methods have been used for morphological studies of the brain at cellular level. A further step towards the description of neuronal population subtypes was the application of immunohistochemical techniques aimed at imaging neuronal structures relying on the expression of neuronal markers (*e.g.* Calbindin or Tuj1-beta for immature post-mitotic neurons, DCX for neuroblasts, and NeuN for mature neurons). Nevertheless the limitations related to the diversity of neuronal populations and the heterogeneous timing of neuronal marker expression, together with the specific localization of the binding sites (*e.g.* nuclear, membrane-bound, or cytosolic), have made single cell morphological studies challenging. The application of virus-mediated gene transfer targeting specific neuronal cell populations (*e.g.* specific transgenic mouse lines) has facilitated the approach to morphologic and functional studies. The use of retroviral labelling in particular has enabled a new method of screening thanks to the possibility of selectively labeling proliferating cells, which allows morphological studies at virtually any stage of neuronal maturation. In this thesis we have used a retroviral vector based on Moloney murine leukemia virus, created from the transfection of capside (CMV-VsVg), viral proteins (CMVpg), and retroviral plasmid (CAG-GFP) (Naviaux et al., 1996). The CAG-GFP gene is a synthetic gene from *Aequorea Victoria* that produces green fluorescent protein (GFP). The retroviral vector is delivered through stereotactic injection and infects only dividing cells within 3 days post injection (Zhao et al., 2006). GFP is expressed in the entire neuronal structure and allows fine analysis of the morphological details. A detailed description of the methodology for retroviral particle production and use is described in Papers II and IV. The infected neurons acquire a morphology similar to pre-existing granule cell neurons by 28 days after fate specification (see (van Praag et al., 2002; Zhao et al., 2006)) making this method particularly suitable for the study of a homogeneous population of mature granule cells representative for the totality of the adult granule cells in the DG. The injection site for 12 mo old mice

was calculated in order to reach the sub-granular zone of the DG in the intermediate portion of the hippocampus, along the dorso-ventral axis, by using the position of the bregma as reference (Paxinos and Franklin, 2004): antero-posterior, -2.6 mm; medio-lateral, +1.75 mm; dorso-ventral, -2.0 mm (from dura).

3.5 SAMPLE PREPARATION FOR IMMUNOHISTOCHEMICAL PROCEDURES

Four weeks after the retrovirus infection, the mice were killed by an overdose of anesthetic (sodium pentobarbital, 150 mg kg^{-1}) and perfused transcardially with ice-cold buffered saline followed by 4% paraformaldehyde. Each brain sample was post fixed with 4% paraformaldehyde and the two hemispheres were differentially processed as follows: the left hemisphere (not injected with virus) was cryoprotected with 15% sucrose, quickly frozen on dry-ice, and 20 μm thick sections were cut with a cryostat. The right hemisphere, which received the retroviral injection, was embedded in 5% TopVision™ Low Melting Point Agarose (Thermo Scientific, Wilmington, DE, USA) and cut in 70 μm thick coronal sections with a vibratome (Leica VT1000s) for confocal microscopy imaging. This procedure was optimized to maximize the use of samples and minimize the number of animals used for ethical considerations.

3.6 ANALYSIS OF HIPPOCAMPAL NEUROGENESIS

To investigate neurogenesis, we estimated the progenitor proliferation and the maturation of newly generated neurons in the DG by immunohistochemical methods.

Two different approaches were used in this thesis: EdU positive and DCX positive cell counting. EdU labeling is a method based on the incorporation of 5–ethynyl–2′–deoxyuridine (EdU; a thymidine analogue) into the DNA of mitotically active cells. Animals were injected i.p. with EdU (50 mg/kg) for one week before sacrifice. EdU was visualized in histological preparation with a fluorescent azide in a copper–catalyzed [3+2] cycloaddition (“Click” reaction) (Zeng et al., 2011) which doesn’t require DNA denaturation. Although used frequently for evaluation of adult neurogenesis, the method is not specific for neurogenesis since it labels every dividing cell (glial cells as well as endothelial cells from blood vessels). Couillard-Despres and colleagues proposed DCX immunohistochemistry as more specific for the evaluation of neurogenic rate as DCX labels only proliferating progenitors committed to neuronal lineage (Couillard-Despres et al., 2005). Similarly, the expression of neuron-specific tubulin epitope lasts until maturation to adult neuron, when the NeuN expression takes over and characterizes the adult neuron population. Therefore the evaluation of adult neurogenesis by means of DCX staining provides a more accurate estimation of the number of newly generated neurons likely to survive until adulthood. In addition, the use of DCX staining prevents the need for daily intraperitoneal injections and therefore reduces the stress in mice. In study I and II both methods are used and show consistent results. For this reason we performed the neurogenesis evaluation presented in the last paper only by DCX staining.

3.7 ANALYSIS OF GR EXPRESSION

The expression of GR was assessed by measuring the fluorescence intensity in the DG from immune-labeled sections. Sections mounted on SuperFrostPlus Microscope Slides (VWR International) and circled with Dako pen were air dried for 10 min at room temperature, rinsed in PBS for 10 min, and subsequently incubated for 2 h in 5% normal guineapig serum (NGS) or normal donkey serum (NDS) in 0.3% Triton in PBS (50 µl/section) . Slides were then incubated for 72 h in GR primary antibody (GR H-300, rabbit polyclonal, Santa Cruz Biotechnology. Inc 1:500) in 0.5% NGS and 0.3% Triton in PBS at 4ºC. After 3 consecutive 5 min washes with PBS, the slides were incubated for 2 h with secondary Ab (Alexa 488) Donkey anti Rabbit (1:200 in 1% BSA and 0.3% Triton) at room temperature. After washing 2 times for 5 min with PBS, we applied nuclear staining with DAPI (1:10,000) for 5 min. After 3 washing of 5 min each slides were mounted with DAKO Fluorescent Mounting media. The intensity of the positive signal was estimated in the granule cell layer (manually delineated), and the background intensity was evaluated in the molecular and polymorph layers of the DG.

3.8 IMAGING AND ANALYSIS

For morphological analyses, all brain sections (12-15/brain) from the right hemisphere through the entire hippocampus were processed for free floating immunohistochemistry for GFP and NeuN. To assess the complexity of 28 days old granule cells, we imaged an average optical thickness of 50 µm (from the slice thickness of 70 µm). Z-series at 1 µm intervals were acquired with a Plan Apochromat 20x/0.75 objective, digital zoom 1.5, on a Zeiss ZEN 2009 LSM 510 META confocal system. A total of 5-12 cells were analyzed from each mouse. For the spine density evaluation, images of GFP-labeled dendritic processes at the outer molecular layer were acquired with z-series at 0.5µm intervals, Plan-Apocromat 63x/1.4 Oil DIC, digital zoom 3. A total of 5 dendritic segments from each mouse were analyzed. All images were analyzed in FiJi (Schindelin et al., 2012). Images of dendritic arborization were deconvolved with Iterative deconvolve 3D plugin and manually traced with Simple Neurite Tracer plugin. The dendritic extension and number of intersections were obtained by automated Sholl analysis (Fig. 3-2).

Sholl analysis counts the number of intersections between dendrites and equally spaced, concentric circles centered on the soma. The spine density was manually calculated on maximum intensity projections of z-series from dendritic segments. Confocal imaging and data quantification were done by the same person, who was blinded to the experimental conditions.

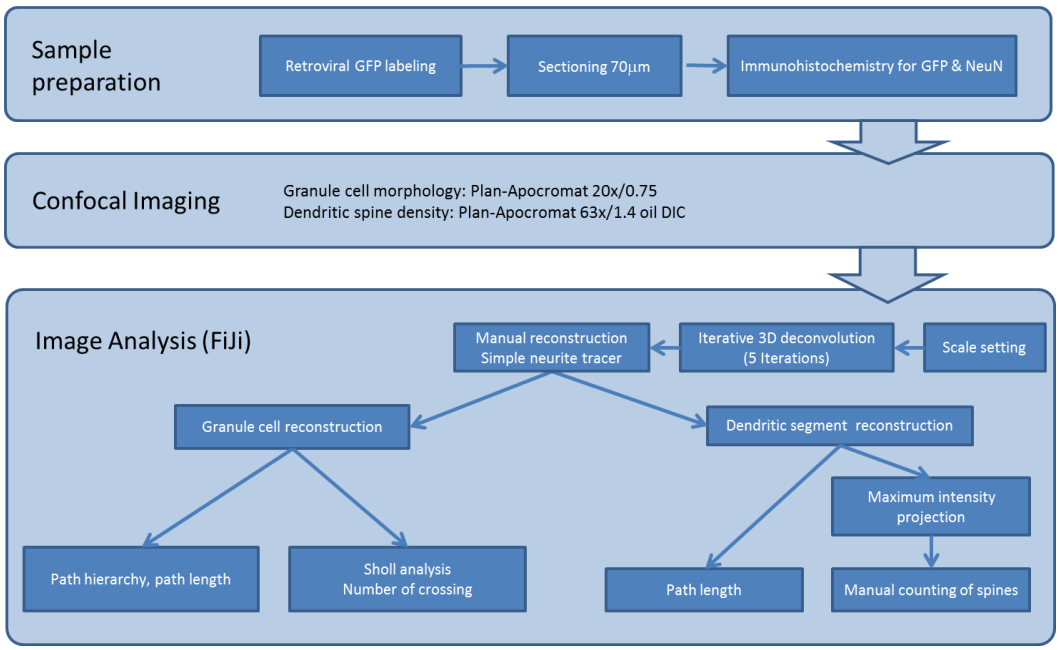

Figure 3-2 Schematic depiction of the workflow for image acquisition and analysis.

3.9 ANALYSIS OF GENE EXPRESSION IN THE HIPPOCAMPUS AND SCN

Hippocampal samples, trimmed from 140 µm frozen coronal sections from the left hemisphere were collected and processed for RNA extraction. The SCN was dissected as a small piece of tissue on ventral extent of the brain section adjacent to the third ventricle, immediately dorsal to the optic chiasm. The accuracy of SCN dissection was confirmed by the high content of AVP mRNA, and extremely low AVP receptor V1b mRNA. RNA extraction was performed using FFPE RNA Purification Kit (Norgen Biotek, Montreal, Canada) according to the manufacturer instruction. Concentration of RNA was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA (1 µg template/sample) was reverse-transcribed into cDNA using 0.5 µg of oligo-dT primer according to the instruction of Maxima first strand cDNA Synthesis kit (ThermoFisher, Wilmington, DE, USA). Amplification reactions were performed on a QS5 System (Thermo Scientific, Wilmington, DE, USA) using 1µl cDNA template, SYBR Green PCR MasterMix (ThermoFisher, Wilmington, DE, USA) and 0.2 µM of each primer, diluted in purified water to a total volume of 12.5 µl. The PCR cycle conditions were: 50ºC for 2min, 95ºC for 10 min, followed by 95ºC for 15s and the annealing temperature for 1 min for 40 cycles. The specificity of the qRT-PCR reactions was evaluated by including a dissociation stage to the melting curve analysis. The data were analyzed by QuantStudio 5 System (ThermoFisher, Wilmington, DE, USA). A table with primers, annealing temperatures, and length of amplification product is available in SI (Paper II). The expression values were normalized against the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) according to the following formula: ΔCT (difference threshold cycles) = CT of target gene-mean CT of housekeeping gene. The relative expression levels (ΔΔCT) were calculated as ΔΔCT = ΔCT exposed –ΔCT controls. To estimate the relative expression regulation we used the formula $2^{-\Delta\Delta CT}$.

3.10 EMBRYONIC CORTICAL NSC CULTURE AND EXPOSURE PROCEDURES

Primary cultures of NSCs were prepared as described in paper III. The cells were obtained from embryonic cortices (n=6–8 per cell preparation) from timed- pregnant Sprague-Dawley rats (Harlan Laboratories, The Netherlands) at GD15 and dissected in HBSS (Life Technologies, Carlsbad, CA, USA). The tissue was mechanically dispersed, and meninges and larger cell clumps were allowed to sediment for 10 min. The cells were plated at a density of 40 000 per cm^2 on a dish pre-coated with poly-L-ornithine and fibronectin (Sigma-Aldrich, Stockholm, Sweden). The cells were maintained in N-2 medium enriched with 10 ng/ml basic fibroblast growth factor (bFGF; R&D systems, Minneapolis, MN, USA) added every 24 h. The medium was changed every other day to keep cells in an undifferentiated and proliferative state. The cells were mechanically passaged via scraping in HBSS. Afterwards, the cells were gently mixed in N-2 medium, counted, and plated at the desired density. Under these culture conditions, NSC doubling time was ~ 20 h. To investigate the heritable effects of DEX on proliferating NSCs, we exposed NSCs to DEX (1 μM) for 48 h, as described earlier. Parental (P1) cells were harvested at the end of the exposure. We then passaged the cells in presence of bFGF but in absence of DEX, to obtain daughter cells (D2 and D3). Mitotically heritable effects were investigated in D3 cells that had never been directly exposed to DEX 72 h after respective passaging.

3.11 GENOMIC DNA EXTRACTION

DNA was prepared using the XL GenDNA extraction module kit (Diagenode, Liège, Belgium) according to the manufacturer's instructions. Quality and quantity of DNA was measured using NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Quant-iT PicoGreen dsDNA reagent and kits (Invitrogen, Paisley, UK).

3.11.1 Extraction of RNA and DNA from DEX-exposed offspring

Newborn mice exposed to DEX in utero were obtained by injecting pregnant dams as descried previously. PND3 pups were killed by decapitation and the brain was rapidly dissected on ice and stored at − 80°C until processing. Next, RNA and DNA were extracted from dissected cortices using RNA and DNA extraction kits as instructed by the manufacturer. RNA was used for the analysis of gene expression by qPCR, while the DNA was used for measuring global DNA methylation and hydroxymethylation as described below.

3.11.2 DNA methylation and hydroxymethylation assay

DNA was prepared using the GeneElute mammalian genomic DNA miniprep kit (Sigma-Aldrich, Stockholm, Sweden) according to the manufacturer's instructions. DNA quality and concentration was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Separately global DNA methylation (5-mC) and hydroxymethylation (5-hmC) were determined using two different quantification kits (Epigentek, New York, NY, USA).

3.11.3 Methyl-DNA immunoprecipitation sequencing, MBD-seq

DNA was sonicated using Bioruptor 200 (Diagenode, Liège, Belgium) at high frequency with 30 s off/ on cycles. The average length of sonicated DNA was 200 bp, which was determined by gel electrophoresis. We used 1.2 μg of sonicated DNA for subsequent MBD2 enrichment using MethylMiner methylated DNA enrichment kit (Life Technologies, Carlsbad, CA, USA). Briefly, first 10 μl of Dynabeads (Life Technologies, Carlsbad, CA, USA) M-280 streptavidin were cleaned by 1 × bind/wash buffer and 3.5 μg of BMD-biotin protein was mixed with clean Dynabeads (Life Technologies, Carlsbad, CA, USA) on a rotating mixer for 1 h. Then DNA fragments were incubated with the coupled MBD-beads overnight at 4°C. After removing non- captured DNA as supernatant, captured DNA was isolated by NaCl gradient elution (0.5 and 1 M). The accuracy of the assay was confirmed by using kit-supplied control DNA. Isolation of hypermethylated (0.5 and 1M) and non-methylated DNAs (supernatant) was confirmed by quantitative real-time PCR analysis using Tsh2b (methylation-specific primer) or Gapdh (non-methylation-specific primer (Diagenode, Liège, Belgium). The recovered DNA was quantified by Qubit (Invitrogen, Paisley, UK) and 50 ng of immunoprecipited DNA was used for library preparation using a kit from New England Biolabs (NEB# E6240S/L, BioNordika Sweden AB, Stockholm, Sweden). Subsequently, the library was analyzed by HiSeq 2000, (Illumina Inc, San Diego, CA, USA). The sequence tags were then aligned to the rat genome (assembly rn4) with the Bowtie alignment tool (http://bowtie-bio. sourceforge.net/index.shtml). To avoid any PCR bias, we allowed only one read per chromosomal position. Next, the peaks (hypermethylated regions) were identified using MACS software (Szabo and Hoffman, 2012; Zhang et al., 2008) and the rat CGIs were downloaded from the UCSC database (http://genome.ucsc.edu). The genome-wide DNA methylation levels were quantified by analyzing mapped sequencing reads as methylation peaks, using the supernatant of each sample as standard background and are referred to as total number of peaks.

3.12 STATISTICAL ANALYSES

All statistical analyses were performed in Statistica™ (Dell Inc. Tulsa, OK, USA). Immobility time in FST was analyzed using a mixed model ANOVA with repeated measures, followed by contrast analysis. Neurogenesis (number of DCX positive neurons) was analyzed using a factorial ANOVA model with interaction terms. Pairwise differences were tested independently using student's t-test. Measures from traced neurons were averaged separately for each subject. For measures of number of branch point and dendritic extension a mixed ANOVA model was tested for the effect of the treatments. The differences between Sholl curves from each group were reported only when significant pairwise differences were found for each consecutive point (pairwise t-test). Gene expression was analyzed using one-sample t-test vs. "no-regulation" value (=1), followed by t-test between groups. Differences are reported as significant for $p<0.05$. The data are shown as average ± SEM.

4 RESULTS AND DISCUSSION

4.1 MODEL ASSESSMENT

The exposure to DEX from GD14 until delivery induced a mild but consistent decrease in intrauterine growth rate (Supplementary Fig. 1A in Paper I). After delivery, the bodyweight was lower in both male and female DEX-exposed mice. The difference was consistent until weaning (PND 21), but disappeared soon thereafter (no significant difference at PND 28; Supplementary Fig. 1B in Paper I).

A battery of tests was run to assess early alterations in spontaneous behavior and anxiety related disorders. The behavioral outcomes of prenatal exposure to DEX, including hyperactivity in the open field and impaired social behavior in the social recognition test, were only present in the male offspring (Supplementary Fig. 2 in Paper I). The sex-differences were in agreement with previous studies (Mueller and Bale, 2008; Weinstock, 2007), therefore we used only the male offspring for the following experiments. The treatment with DEX did not affect mother's behavior measured as maternal care, which if altered, could represent a confounding factor in the interpretation of expected late onset alterations in the offspring. The pup retrieval test was performed at PND 4, upon returning the pups to the cage after culling. The test showed no differences between mothers injected with DEX and controls in the quality of nest building, time spent in the retrieval of the litters to the nest and time spent in nursing. Therefore we can assume that behavioral alterations reflect the persistent effects of developmental exposure to DEX, and are not significantly influenced by differences in maternal behavior.

4.2 LATE ONSET DEPRESSION-LIKE BEHAVIOR

We tested male offspring in the FST at several ages, and found that DEX-exposed mice showed increased immobility time at 12 mo, but not earlier (Fig. 1 in Paper I). The depression-like phenotype was confirmed in the tail suspension test (Supplementary Fig. 3, in Paper I). This result was in agreement with previous findings on affective disorders resulting from prenatal exposure to excess GCs in rodents, although differences in timing of onset and sex appear to be related to the animal model, dose of DEX and timing of exposure (Hauser et al., 2009; Liu et al., 2012; Welberg et al., 2001; Wyrwoll and Holmes, 2012). Nevertheless HPA axis malfunction in the stress response is a common denominator.

4.3 ALTERATIONS IN NEUROGENESIS

We investigated neurogenesis at 12 mo of age and observed that DEX-exposed mice had a lower number of EdU-positive cells in the SGZ (Fig. 2A in Paper I) and less DCX-positive cells in the granular layer of the DG (Fig. 2B in Paper I). The count of DCX-positive cells throughout the DG has been shown to provide an accurate estimate of hippocampal neurogenesis rate (Couillard-Despres et al., 2005). In our studies we found a consistent similar proportion between EdU and DCX-positive cells counts, therefore we adopted DCX staining as preferential method for neurogenesis assessment. Recent studies in rodents have

pointed out a functional segregation of the hippocampus along the dorso-ventral axis (Wu and Hen, 2014) attributing to the ventral portion a preferential involvement in emotional related functions (Leary and Cryan, 2014) and higher sensitivity to GCs (Workman et al., 2015). A preferential action of antidepressants on ventral hippocampus neurogenesis is also suggested (Leary and Cryan, 2014). When we analyzed the effects of DEX along the longitudinal axis of the DG we did not find clear dorso-ventral gradients in neurogenesis (Supplementary Fig. 1 in Paper II), as the decrease in neurogenesis was uniformly distributed. Neurogenesis is a complex process initiated by the activation of quiescent neuronal progenitor cells, and involving gene expression changes, extracellular signaling, neurotrophic factors, neurotransmitters, and epigenetic modifications (Schafer and Gage, 2016). From the genes known to regulate neurogenesis we selected those that are deregulated in depression models and differentially regulated by antidepressants. Notably, some of the selected genes showed persistent alterations in DNA methylation in NSC cultures, as well as cortices from PND3 pups exposed to DEX (Paper III).

The decrease in neurogenesis in DEX-mice was associated with up-regulation of p16 and Cdkn1c, and down-regulation of Klf15 (Krüppel-like factor), three relevant genes known to play key roles in neurogenesis. The cyclin-dependent kinase inhibitor p16 needs to be repressed to maintain the proliferative state of neuronal progenitors, and its up-regulation is a marker of cell senescence (Stein et al., 1999). Interestingly, p16 is persistently up-regulated in NSC cultures exposed to DEX (Bose et al., 2010). Cdkn1c, another cyclin-dependent kinase inhibitor which has growth inhibitory properties (Boonen et al., 2016), is expressed in quiescent progenitor cells and immature neurons and promotes cell cycle exit in NSCs (Furutachi et al., 2013). Klf15 a transcription factor expressed in the ventricular zone during brain development that regulates neuronal differentiation at late stages, inhibits neuronal differentiation when overexpressed, contributing to the maintenance of the NSC population at late embryonic stages (Ohtsuka et al., 2011). This pool of genes was part of a broader list of genes generated by high throughput sequencing of immune-precipitated methylated DNA obtained from NSC exposed to DEX *in vitro* (see Paper III). The up-regulation of p16 and Cdkn1c and the down-regulation of Klf15 in concert could trigger the impairment in hippocampal neurogenesis observed in DEX-exposed mice that develop depression-like behavior. In our experiments, we isolated RNA from tissue homogenates obtained from the entire hippocampus. Therefore the results cannot be ascribed specifically to the adult-born granule cells, but reflect the expression in other cell populations as well. However it should be noted that some genes like Cdkn1c are uniquely expressed in the quiescent progenitors and immature neurons in the DG of the hippocampus (Furutachi et al., 2015, 2013). Further analyses at single cell level (such as promoter methylation) *in vivo* should be considered to specifically address the epigenetic changes induced by DEX exposure.

4.3.1 Alterations in neurogenesis *in vitro*

NSCs show persistent alterations in proliferation and differentiation following DEX exposure (Bose et al., 2010). In addition, they display global DNA hypomethylation, suggesting the

involvement of epigenetic changes. To further investigate the mechanisms of epigenetic modifications induced by DEX, we analyzed the genome-wide DNA methylation pattern in proliferating NSCs isolated from rat embryos. Parent (P1) NSCs, i.e. directly exposed to DEX, and daughter cells (D3), i.e. never directly exposed to DEX, exhibited stable alterations in DNA methylation: about 64% of the methylated peaks were preserved between P1 and D3 (Fig.1 in Paper III). The hypermethylated DNA sequences were located in promoter regions of genes known to regulate cell proliferation, differentiation, migration, and cell senescence (Supplementary tables S10 and S11 in Paper III) like FOXO3a, Cdkl5 and Gsk3b. In agreement with these results, the expression of enzymes regulating DNA methylation, such as Dnmt3a and Tet3 was altered. We further investigated the role of GR signaling and found that a key alteration was the GR-dependent Tet3 down-regulation. The functional relevance of Dnmt3a and Tet3 regulation was confirmed by the changes in 5-mC and 5-hmC levels. We validated these mechanisms *in vivo* by analyzing cerebral cortices isolated from PND 3 mouse pups exposed to DEX *in utero*. Similar to NSCs in culture, the Dnmt3a down-regulation and Tet3 up-regulation were accompanied by a decrease in 5-mC and an increase in 5-hmC levels. These data provide an indication about the class of genes most probably involved in the alterations observed *in vivo*.

4.4 ALTERATIONS IN NEURONAL MORPHOLOGY

Decreased complexity in dendritic arborization has been described in experimental models of depression, as well as in post-mortem material collected from depressed patients (Llorens-Martín et al., 2016; Qiao et al., 2016; Ren et al., 2015), and there is evidence that effective antidepressant treatments are accompanied by restoration of dendritic arborization (Morais et al., 2014; Seo et al., 2014). To investigate the effects of DEX on the morphology of newly generated neurons, we used retrovirus-mediated labeling with GFP. The neurons in the granule cell layer of DEX-exposed mice displayed an overall decrease in the dendritic arborization complexity, as measured by the total dendrite extension and number of terminal dendrites (Fig. 3A in Paper II). In addition, the dendritic alterations were accompanied by a decrease in spine density on distal dendritic segments (Fig. 3B in Paper II).

We further investigated the qualitative alterations in dendritic arborization by means of Sholl analysis (Fig. 3C in Paper II). We found that DEX-exposed mice have a higher number of crossings in proximal segments (between 10 and 50 µm from the soma, where the primary dendrites are localized), but a lower number of crossings in the distal region (between 90 and 260 µm from the soma, where terminal dendrites are localized) as compared to controls. The configuration of dendritic arborization that we found in DEX-exposed mice (*i.e.* enhanced proximal branching, but lower number of terminal dendrites) is consistent with a V-shape phenotype (Llorens-Martín et al., 2016), in contrast to the more common Y-shape (see Fig. 4A in Paper II; Fig. 4-1).

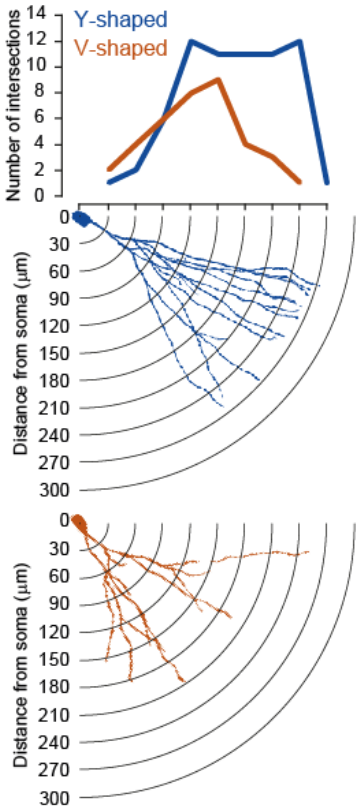

Figure 4-1 Illustration of Sholl analysis. The number of intersections between the concentric spheres and the dendritic tree are plotted as a function of distance from the soma to generate the Sholl curves. Qualitative differences between Y-shaped and V-shaped phenotypes (bottom panels) are readily depicted by distinctive Sholl curves (top panel).

We assessed the occurrence of V-shaped neurons and found that DEX-exposed mice displayed about 1/2 V-shaped neurons, as compared to about 1/4 in controls (Fig. 4B). A high frequency of neurons with a V-shaped phenotype has been described so far only in severe disorders, namely in post-mortem brain from Alzheimer's Disease patients (Einstein et al., 1994; Flood et al., 1987), or in experimental models of neuroinflammation (Lee et al., 2015; Llorens-Martín et al., 2016). This conformation is therefore probably indicative of severe disorders and can potentially lead to hippocampal function impairment (McAllister, 2000). In V-shaped neurons, recurrent excitatory connections from CAF are more frequent than in Y-shaped neurons (Förster et al., 2006). Conversely, entorhinal cortex inputs, which normally contribute to the dendritic maturation of granule cells, reach fewer distal dendrites as compared to Y-shaped neurons (Frotscher et al., 2000). Therefore, the V-shaped morphology favors excitatory signals from the ipsilateral and contralateral granule cells over those from the EC. This arrangement is reminiscent of the epileptic condition in which mossy fiber collaterals establish aberrant synapses with granule cell dendrites. It has been proposed that these aberrant projections alter the balance between inhibitory and excitatory input to the DG. However, we did not find malpositioning of granule cells in the DG, which typically accompany epilepsy (Förster et al., 2006).

Neuronal morphology of adult born neurons responds to intrinsic programming in the initial stages, as well as neurotransmitter-programming mechanisms and molecular signaling from the pre-existing neuronal architecture. The expression of genes regulating neuronal maturation is not restricted to neuronal precursors, but is found also in adult neurons and non-neuronal cells (*e.g.* astrocytes (Terrillion et al., 2017)). In addition, the transition to more mature and excitatory granule cells with higher dendritic tree complexity is regulated by

innervation from extra-hippocampal regions (*e.g.* EC) and intra-hippocampal inter-neuronal fibers (Crowther and Song, 2014; Frotscher et al., 2000). Variations in morphology of new-born granule neurons are not only dependent on physiological conditions but also on detrimental stimuli such as stress, dietary factors and drugs of abuse, as extensively reviewed by Llorrens-Martìns and colleagues (Llorens-Martín et al., 2016). Post-mitotic regulation in the expression of several plasticity related genes characterize the highly dynamic adaptations that granule cells, together with pyramidal cell population from CA implement in order to properly respond to the ever-changing environment or to insults of various nature. In the screening for genes likely to be involved in the alteration observed we selected some of those known to be sensitive to GCs excess and also responsive to antidepressant treatment.

We found that the expression of TrkB (receptor for BDNF; (Li et al., 2009; Martínez et al., 1998)), disrupted in schizophrenia 1 (DISC1; regulator of dendritic arborization), and GAP-43 (regulator of cell cycle, neuronal differentiation, maturation and plasticity (Mani et al., 2001; Zhao et al., 2009)) was down-regulated in DEX-mice. The alterations in morphology in DEX-mice are consistent with the down-regulation of GAP-43 and TrkB expression. GAP-43 regulates neuronal differentiation and dendritic growth (Chao et al., 1992; Mani et al., 2001; Yaniv et al., 2008; Zhao et al., 2009), and has been shown to be down-regulated in post-mortem brain of patients with MDD (Müller et al., 2001). Moreover, its expression has been shown to be up-regulated following antidepressant treatment with imipramine (Sairanen et al., 2007; Yaniv et al., 2008).

DEX exposure was also associated with downregulation of Reelin (Reln) a glycoprotein regulating granule cells' migration in DG and dendritic complexity (Förster et al., 2006). The alterations in the pattern of dendritic arborization of granule cells in DEX-mice are compatible with the down-regulation of DISC1 and Reln. Both genes are highly expressed in the adult hippocampus, where they regulate the maturation of new-born granule cells, and have been associated with psychiatric conditions such as schizophrenia (Kempermann et al., 2008) and depression (Lussier et al., 2013). DISC1 appears to be particularly relevant in our model, because DISC1-Q3IL mutant mice display depression-like behavior and treatment with FLX or DMI ameliorates the phenotype (Lipina et al., 2012). In addition, DISC1 down-regulation increases the number of primary dendrites (Lee et al., 2015), thereby promoting the occurrence of V-shaped granule cells (Llorens-Martín et al., 2016). Similarly, the down-regulation of Reln in the DG has been proposed as a mechanism through which GCs lead to progressive development of depression like behavior (Fenton et al., 2015).

A particularly relevant finding in relation to the lack of response to FLX is the down-regulation of TrkB gene expression in the hippocampus of DEX–exposed mice. BDNF (whose expression is not altered in our model; unpublished data) stimulates neurogenesis via the TrkB receptor by activating the MAPK/CREB pathway (Li et al., 2009). While the response to antidepressants is typically associated with BDNF up-regulation (Lindholm and Castrén, 2014), TrkB knock-out mice display impaired neurogenesis and SSRI-resistant depression (Li et al., 2009; Yan et al., 2016) similar to our DEX-exposed mice. TrkB is also

required for proper hippocampal innervation by commissural axons, as well as for synaptogenesis and maturation of hippocampal connections (Martínez et al., 1998).

4.5 ANTIDEPRESSANT TREATMENT

We selected FLX based on our previous study (Onishchenko et al., 2008) and on the established effectiveness of SSRIs in models of depression induced by prenatal stress (Nagano et al., 2012; Rayen et al., 2011). Treatment with FLX for 21 days before repeating the FST did not affect the immobility time in DEX-exposed mice, and did not have any significant effect on neurogenesis.

It has been previously shown that the effect of FLX on depression and neurogenesis requires rhythmic corticosterone secretion in rodents, suggesting that alterations in HPA axis regulatory loop known to be associated with prenatal stress, may contribute to FLX failure (Huang and Herbert, 2006). Indeed, when we investigated the diurnal rhythm of GCs secretion by measuring the concentration of GCs metabolites in feces DEX-exposed mice exhibited lower levels of corticosterone metabolites and did not show significant diurnal oscillations (Fig. 2C in Paper I).

HPA axis regulation, including the circadian fluctuations in GCs secretion, involves hippocampal GR expression in the feedback loop (Jankord and Herman, 2008; Sapolsky et al., 1984). Twelve month-old DEX-exposed mice display significant downregulation in hippocampal GR expression (Fig. 2D and Supplementary Fig. 4 in Paper I), in agreement with earlier reports (Levitt et al., 1996; Welberg et al., 2001).

The SNRI antidepressant DMI has been previously shown to be effective in depression models resistant to FLX (Guo and Lu, 2014; Holmes et al., 2002; Yu et al., 2012). Both FLX and DMI have been reported to restore neurogenesis (Chen et al., 2006; Guirado et al., 2012) as well as the complexity of dendritic arborization and the density of dendritic spines in the hippocampus (Laifenfeld et al., 2002; Norrholm and Ouimet, 2000). Unlike FLX, which relies on rhythmic GR activation for its antidepressant effects (Huang and Herbert, 2006), DMI enhances GR nuclear translocation and gene transcription via a steroid-independent mechanism (Chen et al., 2001; Huang and Herbert, 2006). In addition NE-receptor mediated intracellular cascades have been described to promote neurogenesis, proliferation and neurite growth by regulating the expression of genes involved in neuronal sprouting and differentiation (Laifenfeld et al., 2002).

When we re-tested the mice for FST after 4 weeks of treatment with DMI, the immobility time was decreased significantly only in DEX-mice (Fig. 1 in Paper I). We also observed that DMI increased the number of DCX-positive neurons in DEX-exposed mice (Fig. 2 in Paper II). The restoration of neurogenesis to control levels was accompanied by Cdkn1c, p16 and Klf15 normalization (see Fig. 5 in Paper II). Interestingly, the expression of Mbd1 (which, together with BMi1 and MLL1, controls the timing of cell cycle exit and initiates neuronal differentiation in the adult brain (Mateus-Pinheiro et al., 2011)) was not affected by DEX, but up-regulated by DMI treatment only in DEX-mice. Adult neurogenesis is epigenetically

regulated by a battery of genes like Mbd1. Mbd1 binds the promoter region of the gene encoding the mitogen Fibroblast Growth Factor 2 (FGF2), controlling its expression in NSC and regulating the exit from proliferative cycle towards differentiation (Mateus-Pinheiro et al., 2011). DMI promoted Mbd1 up-regulation, which could contribute to the positive effects on neurogenesis observed in the DEX-exposed mice after DMI treatment.

DMI also had a remarkable impact on the adult-born granule neurons morphology. After DMI treatment, the proportion of V-shaped phenotype decreased to control levels in DEX-exposed mice, while no significant changes were detected in controls (Fig. 4 B in Paper II). Overall, the effects of DMI on spatial distribution of dendritic arborization and spine density (see also Supplementary Fig. 2 in Paper II) were associated with a differential regulation of the plasticity related genes analyzed. Indeed we found that the expression of GAP-43 and TrkB is restored to control levels after DMI treatment in DEX-exposed mice. Although no direct interaction of NE and TrkB has been reported, Yaniv et al. describe that the activation of alpha2-adrenergic receptors by NE induces the expression of plasticity related genes (*e.g.* GAP-43) through the MAPK/ERK/CREB pathway (Yaniv et al., 2010, 2008). Thus, the restoration of neurogenesis and dendrite growth by DMI can be the result of TrkB-mediated and NE signaling converging on CREB activation.

4.6 CIRCADIAN RHYTHM ALTERATIONS

4.6.1 Alterations in circadian entrainment of spontaneous activity

4.6.1.1 Rhythmicity in spontaneous activity

Spontaneous activity is mainly regulated by circadian photic entrainment and requires metabolic proper response in order to predict regular cyclic changes in the environment to the internal clock. The diurnal fluctuation in GCs serve this fundamental hormonal adaptations (Kiessling et al., 2010; Weibel et al., 2002). Therefore we asked whether the blunting in GCs fluctuation in DEX-mice could reveal anomalies in circadian entrainment in steady-state conditions (LD cycle or DD), as well as in response to circadian re-entrainment (restoration of LD after DD; forced synchronization; Fig. 3A in Paper I). In spontaneous activity, this is illustrated by the acrophase (the time at which the peak of a rhythm occurs (Cornelissen, 2014)) occurring before ZT 18 (*i.e.* the middle of the dark phase) in the context of steady entrainment with 24 h circadian period. In contrast, forced synchronization transiently increases the amplitude of circadian oscillations and delays the acrophase to around ZT 18. DEX-exposed mice displayed larger amplitude than controls during steady entrainment already from 1 mo of age, and showed no significant difference between steady entrainment and forced synchronization (Fig. 3B in Paper I). The acrophase in DEX-exposed mice occurred close to ZT 18 at all ages tested, and was significantly delayed by forced synchronization only at 1 mo (Fig. 3C in Paper I). On the other hand, the acrophase of spontaneous activity in control mice occurred consistently before ZT 18 under steady entrainment conditions, and was significantly delayed by forced resynchronization at 1, 3, and 5 mo (Fig. 3C in Paper I). This suggested that photic entrainment has a particularly

strong effect in DEX-exposed mice. Moreover, the onset and offset of active phase virtually coincided with the onset and offset of the dark phase (Fig. 3D in Paper I). Therefore, DEX-exposed mice appear to have shorter active phase duration at all ages tested (Fig. 3E in Paper I).

4.6.1.2 Variability in spontaneous activity

The central clock, located in the SCN controls the intrinsic rhythmicity in spontaneous activity at multiple timescales (*i.e.* circadian as well as ultradian oscillations) (Hu, 2009; Hu et al., 2012). The variability of spontaneous is an intrinsic feature of the regulation of spontaneous activity by the SCN, and should therefore not vary significantly between any two steady-state conditions (*e.g.* free-running and constant LD cycle).

In our model, the mice exposed to DEX tend to have higher scaling exponent under constant photic entrainment (LD), which suggests with a pattern where diurnal fluctuations are the dominant cyclic component. Abrupt transitions between steady states induce transient changes in scaling exponent, presumably due to a strong diurnal rhythm (see Fig. 5B in Paper I). This effect reach significance in controls only at the ages of 1 and 3 mo, while in DEX-exposed mice it was consistently detected at all ages tested. In contrast, DEX-exposed mice displayed consistently higher scaling exponent under constant photic entrainment as compared to free-running conditions. This is consistent with a pattern of activity dominated by diurnal fluctuations and lead to the investigation of the response to an abrupt change in LD cycle.

4.6.2 Phaseshift challenges the ability to re-entrain

We analyzed the spontaneous activity in response to a 6 h phase advance at the age of 6 mo (Fig. 4A in Paper II). It is worth to mention that the transition to dark allows, but does not trigger the onset of activity, in contrast to light onset, which suppresses the activity and directly resets the central clock (Challet, 2007; Golombek and Rosenstein, 2010). Thus, advancing the onset of the dark challenges the ability of mice to re-entrain.

We observed that the acrophase is advanced by 6 h immediately after the phase advance in the LD cycle (Fig. 4B in Paper I). When we analyzed the onset and the offset of the active phase in relation to the LD cycle we found that the onset of active phase coincides with the beginning of the dark phase immediately after the phase advance of the LD cycle in DEX-exposed mice. In contrast, the onset of activity is lagging behind the onset of the dark phase in control mice (Fig. 4C in Paper I; Fig. 1C in Paper IV). We confirmed these findings in subsequent phaseshift experiments, where we show that DEX-mice typically re-entrain within 1 day after the phase advance, as compared to about 3 days in controls (Fig. 1C in Paper IV). Altogether, these data suggest that the diurnal rhythms in spontaneous activity in DEX-exposed mice are more rigid in relation to the LD cycle.

4.6.3 Synchronization of peripheral oscillators by AVP

We analyzed the expression of core clock genes Bmal1 and Per1 in SCN samples from animals killed at the peak and at the trough of their expression (*i.e.* early subjective day and early subjective night (Ono et al., 2017). DEX-exposed mice displayed robust circadian fluctuations in Bmal1 and Per1mRNA expression, similar to controls (Fig. 2A in Paper IV). This confirmed that the alterations in entrainment of spontaneous activity in DEX-exposed mice are due to altered photic entrainment, and not to SCN dysfunction in clock genes regulation.

AVP is the main output from SCN (Kalsbeek et al., 2010) and plays a critical role in driving peripheral oscillators, as well as other brain regions, including the hippocampus, through HPA axis rhythmic activity (Buijs et al., 1999). Within the SCN, AVP signaling is critical for the synchronization of neuronal firing patterns, and decreased AVP signaling in the SCN renders the self-sustained oscillation more sensitive to perturbations, such as photic re-entrainment (Yamaguchi et al., 2013). Accelerated re-entrainment ("resistance to jet-lag") has been described in models of impaired AVP signaling in the SCN (Mieda et al., 2015; Tsuji et al., 2017; Yamaguchi et al., 2013). We measured the expression of AVP and we found it was down-regulated in DEX-mice, both at the peak, and at the trough of its expression (Fig. 2B in Paper IV).

This observation indicates that a decrease in AVP signaling could reflect a decreased drive on HPA axis as well as on hippocampus as part of the stress response system. To confirm our hypothesis we measured GR expression in the hippocampus and found a down regulation in DEX-exposed mice. Moreover, the analysis of clock gene expression in the hippocampus revealed a reduction in circadian oscillations and a phase de-synchronization of Bmal1 expression from SCN oscillations. This is supported by the state portraits (Fig. 3B in Paper IV), which show that naïve DEX-exposed mice do not display a pattern reminiscent of coupled oscillations.

4.6.3.1 Clock genes expression and hippocampal neurogenesis

Oscillations in circulating GCs control hippocampal neurogenesis also by driving the expression of clock genes, particularly Bmal1 and Per1 (Bouchard-Cannon et al., 2013; Conway-Campbell et al., 2010; Kimiwada et al., 2009; Segall and Amir, 2010). We observed that the expression of clock genes in the hippocampus is altered in depressed DEX-exposed mice (*i.e.* at the age of 12 mo) (Fig. 2E in Paper I). Interestingly similar alterations in clock gene expression were also displayed at 3mo of age. We further refined the analysis of clock gene expression, and investigated the synchronization of a core clock gene (Bmal1) between the DEX and control running under the same LD conditions. We sampled hippocampi at different time points to depict the discrete distribution of expression over time. The analysis of coupling was performed by plotting the relative expression of Bmal1 (Fig. 3A; Paper IV) within the different groups while accounting for the temporal sequences of sampling. Bmal1

expression in DEX-exposed mice was substantially different from controls, (Fig. 3 in Paper IV) suggesting a different pattern of Bmal1 expression under the same LD conditions.

4.6.4 DMI treatment restores entrainment in DEX mice

Chronic light deprivation apparently impairs specifically the NE- LC system (Gonzalez and Aston-Jones, 2008), and DMI is effective in reversing the behavioral effects associated with light deprivation (González and Aston-Jones, 2006). In addition, selective pharmacological lesion of NE projections from LC have a significant effect on circadian rhythms only in steady entrainment (*i.e.* constant LD cycle), but not in free-running (*i.e.* constant darkness, DD) conditions (González and Aston-Jones, 2006). This suggests that NE modulates diurnal entrainment rather than altering the intrinsic SCN function. Naïve DEX-exposed mice re-entrain within 1 day after the phase advance, as compared to about 3 days in controls (Fig. 1B, C in Paper IV), and re-entrainment was delayed in DEX-exposed mice after 28 days of treatment with DMI (Fig. 1B, C in Paper IV).

For psychiatric disorders, the proposed mechanism is the desynchronization of molecular clocks from the SCN, rather the dysfunction of SCN *per se* (Bering et al., 2017; Li et al., 2013; Menet and Rosbash, 2011) (reviewed recently by (Schibler et al., 2015)). Here we show that the expression of core clock gene Bmal1 in the hippocampus and the SCN is desynchronized in mice exposed to DEX *in utero* (Fig. 3 in Paper IV), which may account for the blunted oscillations in clock genes in the hippocampus of depressed mice (Paper I). We found that the synchronization of the hippocampal clock with the SCN was recovered after DMI treatment (Fig. 3 in Paper IV). The HPA axis regulatory loop is required for the synchronization of slave oscillators with the SCN (Kalsbeek et al., 2010). In DEX-exposed mice, DMI treatment potentiated the drive of SCN on HPA axis by up-regulating AVP expression in the SCN (Fig. 2C in Paper IV; see also (Cagampang et al., 1994; Vacher et al., 2003)), and increased the hippocampal sensitivity to CGs signaling by up-regulating the expression of GR (Fig. 2D in Paper IV). Notably, chronic DMI treatment has a direct effect on GR up-regulation, translocation and steady-state levels in the hippocampus (Lai et al., 2003; Okugawa et al., 1999; Paul Rossby et al., 1995), suggesting that DMI treatment may have persistent effects.

4.7 DMI TREATMENT PREVENTS THE ONSET OF DEPRESSION

We tested the possibility to intervene at early stages of circadian alterations in order to prevent the occurrence of depression-like behavior at 12 mo. Therefore we tested whether antidepressant treatment with DMI at 6 mo could prevent the onset of depression-like behavior and the associated alterations in hippocampal neurogenesis that DEX-exposed mice develop at 12 mo.

Treatment with DMI at 6 mo prevented the increase in immobility time at 12 mo in DEX-exposed mice (Fig. 4 in Paper IV). Similarly, neurogenesis in 12 mo-old mice treated with DMI at the age of 6 mo was not decreased as compared to controls (Fig. 5B in Paper IV). In DEX-exposed mice treated with DMI also the proportion of V-shaped neurons and the

pattern of dendritic arborization were not different from controls (Fig. 5E in Paper IV). The steady effects of DMI on GR expression and activity (Lai et al., 2003; Okugawa et al., 1999; Paul Rossby et al., 1995) could provide a mechanism for the long-term protective effect of DMI. The hippocampal expression of GR was up-regulated following DMI treatment at 6 mo, therefore we can speculate that the persistent up-regulation of GR leading to the restoration of coupling between molecular clocks plays a critical role in preventing the onset of depression in DEX-exposed mice.

4.8 IMPAIRED ENTRAINMENT IS ALSO PRESENT IN PRIMARY SKIN FIBROBLASTS

Fibroblasts isolated from adult DEX-exposed mice displayed smaller amplitude of oscillations in Bmal1 mRNA expression (Fig. 5C in Paper I). This indicates that while the expression of Bmal1 can be synchronized across the fibroblast cell population, the cross-synchronization dissipated faster in the fibroblasts derived from DEX-exposed mice, and is consistent with the facilitated circadian re-entrainment we observed in spontaneous activity.

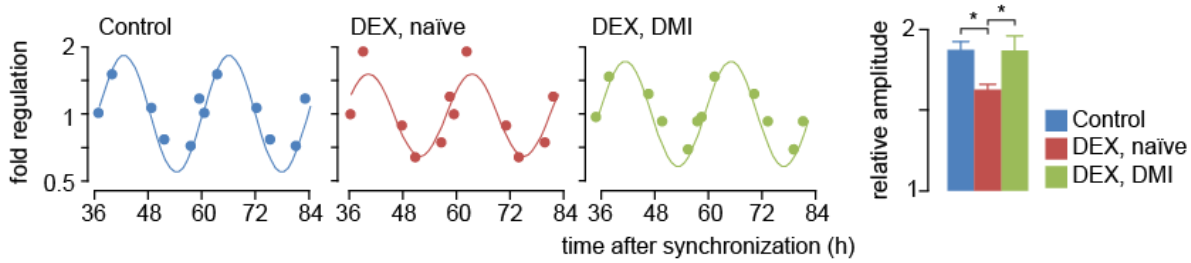

Figure 4-2 Oscillations in Bmal1 expression in primary skin fibroblasts. The cells were isolated form skin samples collected from mice (N=3-4/group) aged 6 mo. The expression of clock genes was synchronized with a pulse of DEX, and the time of harvesting is indicated on the horizontal axis. The data points are double-plotted to visualize the circadian pattern of fluctuations. The amplitude of oscillations was estimated using COSINOR analysis (right panel). The amplitude in Bmal1 expression oscillations was decreased in fibroblasts isolated from naïve DEX-exposed mice, but was restored to control levels in cells isolated from DEX-exposed mice treated with DMI.

In a preliminary experiment, we found that fibroblasts harvested from DEX-mice treated with DMI showed amplitude of oscillations in Bmal1 mRNA expression similar to controls (Fig. 4-2). This result confirmed the efficacy of DMI to restore the ability of the molecular clock machinery to entrain to external synchronizing stimuli.

5 CONCLUDING REMARKS

Epidemiological and experimental studies have shown that alterations in the intrauterine programming occurring during critical periods of development have adverse consequences in later life. Unfavorable prenatal factors induced by stress, childhood abuse/neglect or exposure to neurotoxic food contaminants, can cause epigenetic changes in early life, which may lead to mood disorders in adulthood, as shown in humans and rodents. Specifically, clinical studies have shown a correlation between intrauterine growth retardation, which is commonly associated to high levels of GCs glucocorticoids, and the development of ADHD and depression. The general objective of this thesis project was to investigate the long term consequences of a deranged prenatal hormonal milieu induced by excess glucocorticoids.

Using mice exposed to DEX *in utero* as experimental model, we found that prenatal exposure to DEX induces late onset depression-like behavior in 12 mo old male mice, associated with impaired adult neurogenesis and morphological alteration of adult born granule cells in the hippocampus. Concordantly, the expression of relevant genes controlling neuronal plasticity in the hippocampus is also altered, likely due to epigenetic modifications. DEX-exposed mice also display blunted oscillations of circulating corticosterone and decreased GR expression in all hippocampal cell subpopulation. Moreover, DEX induces alterations in circadian entrainment of spontaneous activity, which appear long before the onset of depression-like behavior. Along with the abnormal entrainment, the coupling of oscillations in the expression of core clock genes between the SCN and the hippocampus is also disrupted.

In the attempt to reverse the depression-like phenotype, we tested two different classes of antidepressants: SSRI (FLX) and SNRI (DMI). FLX treatment has no effect on depressed DEX mice, probably as a consequence of weaker glucocorticoids oscillations. In contrast, DMI has a positive effect not only on depression-like behavior, but also on neurogenesis and neuronal morphology. The mechanism behind the effects of DMI may be linked to the enhanced GR function facilitating GCs intracellular transportation and GR nuclear translocation, as well as the restoration of TrkB gene expression. DMI also promotes the restoration of circadian entrainment of spontaneous activity, which is associated with a normalization of AVP expression in the SCN, as well as with the recovery of the coupling between the molecular clocks in SCN and hippocampus. Of particular interest is the analysis of the molecular clock function in skin fibroblasts that exhibits alterations similar to the ones observed in the central nervous system: they occur before the onset of depression-like behavior, and are reversed by DMI treatment. Altogether, our results support and strengthen the evidence that the prenatal environment plays a critical role in the onset of neurodevelopmental disorders and that epigenetic mechanisms may be involved.

MDD has been recognized by the World Health Organization as a major cause of disability. Worldwide, the prevalence is estimated to about 10% in the general population. The most commonly prescribed antidepressants, such as SSRI, take several months to show beneficial effects, and symptom remission is achieved in only 30-40% of MDD patients. In addition, the

identification of the effective therapy by trial-and-error is a very long process, during which the patients may lose hope and run a significantly increased risk of suicide attempts. Therefore, the identification of drugs that are likely to be effective before starting the treatment is critically important to reduce the burden on both the individual and society. Our findings suggest that alterations in circadian entrainment provide potential biomarkers for identifying subjects at risk of developing depression or depression relapse, as well as to predict the response to antidepressant treatment.

6 ACKNOWLEDGEMENTS

This work was supported by Swedish Research Council (VR), The Brain Foundation (Hjärnfonden), Karolinska Instutet Funds for Doctoral Education (KID). The animal experiments have been performed in the NBR facility funded by Knut and Alice Wallenberg Foundation.

This thesis is the result of the intense collaborations and interactions with a number of people which, even if not always present during the postgraduate period, contributed significantly to its accomplishment.

I would here like to thank:

Sandra Ceccatelli, my main supervisor; she wisely guided my work through this project, helping and challenging me scientifically and personally, and providing a stimulating environment for my growth as a scientist.

Stefan Spulber, my co-supervisor, for supervising me on daily basis, encouraging me in adopting new strategies and formulating appropriate scientific questions. His outstanding knowledge in virtually any scientific field and his tireless work have been fundamental in the accomplishment of this project.

Marilena Raciti, for her competent help and technical advice in setting many of the experiments performed in this thesis. Her contribution has profoundly and positively affected the course of the studies presented.

Natalia Onishchenko, for introducing me to new techniques and patiently encouraging and assisting my first steps into the project, with open minded attitude. **Raj Bose**, for his collaboration in Paper III and **Karin Edoff**, for being always available for technical support during my first months in the lab. **Caitlin DuPont**, for her technical and administrative support and for her positive and pleasant attitude in the lab. **Elena Paci**, **Cristina Battagli**, **Ida Eriksson**, and **Osiris Ointa**, for their help in performing some of the experiments and data analysis. Without them I would still be injecting, pipetting and counting cells by now! **Jeffrey W. Sall**, for his contribution and constructive comments to the revision of the thesis. Personnel from the animal facility for being helpful and taking good care of our animals.

Gilberto Fisone, for his scientific as well as personal support, always with his unique sense of humor.

Julien Bouvier, Peter Löw, and **Carmelo Bellardita** from Ole Kiehn laboratory; they have helped me with competence, material s and equipment for a side project (CLARITY) that for various circumstances did not make it into this thesis. Nevertheless their support has been very valuable to me.

I would also like to thank:

Annamaria Vezzani, my supervisor during my master degree, for teaching me the basics of the scientific method in Mario Negri Institute, and for opening new perspectives by sending me to Sweden, back in the day. **Gino Cervo**, for hosting me in his laboratory in Mario Negri Institute when I left my position in a pharmaceutical company in Italy, allowing me to step back in research environment. **Alessandro Orrù**, for patiently re-introducing me to the lab-work practice and pharmaceutical research.

Thomas and Karina Sakmar and, for their kind friendship and for providing a familiar environment here in Sweden.

And of course, I would like to thank **Laura**, for encouraging me to start this venture, trusting my potentials, and dedicating some extra time to our kids during my "absence". **Chiara** and **Mattia**, my beloved kids, for their understanding during tough times and their loving support during my down moments.

Walter and **Marisa**, my dear parents, for accepting and supporting our choice to leave Italy, renouncing (with heavy hearts) the company and the hugs of their beloved grandchildren.

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