TRANSGENERATIONAL EPIGENETIC INHERITANCE OF THE STRESS RESPONSE IN RATS

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Dedicated to my parents,

Nataliya and Mykola,

and to my grandparents,

Nina and Mykola,

Kateryna and Oleksandr.

ABSTRACT

Recent human and animal studies indicate that stressful experiences *in utero* may have long-term consequences on the future mental wellbeing of the offspring across several generations. Epigenetic mechanisms, such as microRNA expression and DNA methylation are prone to change in response to hostile environmental factors. The main goal of this study was to investigate transgenerational changes in gene and microRNA expression, as well as in DNA methylation patterns in the placenta and developing brain in response to stress in a rat model. Our results demonstrated that ancestral exposure to stress during gestation causes significant changes in gene expression as well as epigenetic changes in placenta and embryonic cortex in the F2 and F3 generations. In addition, our results showed that assessing changes in placenta can be used to predict pathological processes of the newborn brain, thus providing an opportunity for early diagnosis of neurological and psychiatric diseases.

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ACTH - adrenocorticotrophic hormone
AD - autism disorder
ADHD - Attention deficit hyperactivity disorder
ALS - Amyotrophic lateral sclerosis
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA - analysis of variance
AVP - arginine-vasopressin
BBB - blood-brain barrier
BPA - bisphenol A
CNS – central nervous system
CORT - glucocorticoids (cortisol in primates or corticosterone in most rodents)
CRH - corticotropin-releasing hormone
DAVID - Database for Annotation, Visualization and Integrated Discovery
DNMTs - DNA methyltransferases
E21 – embryonic day 21
ECM – extracellular matrix
EDC - endocrine-disrupting chemical
FC – fold change
FE – fold enrichment
G - gestational day
GABA - gamma-aminobutyric acid
GnRHs - gonadotropin-releasing hormones
GR – glucocorticoid receptors
HAB – high anxiety-related behaviour
HPA - hypothalamic-pituitary-adrenal
IAP - intracisternal A particle
IGF - insulin-like growth factor
IUGR - intrauterine growth restriction
KEGG – Kyoto Encyclopedia of Genes and Genomes
LAB – low anxiety-related behaviour
LG-ABN - licking/grooming and arched-back nursing
LTD – long-term depression
LTP - long-term potentiation
MAPK - mitogen-activated protein kinase
MBDs - methyl-CpG binding proteins
MD - major depression
MR – mineralocorticoid receptors
mTOR - mechanistic target of rapamycin
NMDA - N-methyl-D-aspartate
PCO - polycystic ovarian disease
PCR – polymerase chain reaction
PGS - primordial germ cells
PMDs - partially methylated domains
PNN - perineuronal net
PO - parental generation
POI - primary ovarian insufficiency
PVDF - polyvinylidene fluoride
PVN - paraventricular nucleus
SDS - sodium dodecyl sulfate

SGP - slow growth period
SNARE – soluble N-ethylmaleimide sensitive factor attachment protein receptor
TCA - citrate cycle
TGF-beta - transforming growth factor beta
TJs - tight junctions
TSA - trichostatin A

Chapter 1: General Introduction

Stress-induced Perinatal and Transgenerational Epigenetic Programming of Brain Development and Mental Health

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Introduction

The stressful childhoods of our forebears may influence our personality and mental health by altering epigenetic regulation of gene expression in the brain. Notably, such programming by stress may significantly affect the risk of anxiety and depression in the human population. About half the world's population is afflicted by depression, anxiety disorders, or other mental illnesses at some point in their lives, which affects people's ability to function in everyday life and generates a serious economic burden to society. The causes of mental illnesses are poorly understood, however, striking evidence suggests that they may be influenced by stress in previous generations (Franklin et al 2010). In fact, stress may be the single most potent factor in the vulnerability to mental illness (Kinney et al 2008b). This is opposed to resilience, which represents the ability to withstand stress and its health risks by adapting and overcoming adversity (Franklin et al 2012). Transgenerational epigenetic inheritance, which in part manifests itself in altered stress response in the progeny of stressed parents, may critically determine both, stress vulnerability and resilience, thus acting as a maladaptive or an adaptive mechanism.

In this review, we will address the epigenetic mechanisms including changes in the level of non-coding RNAs, DNA methylation and histone modifications that may potentially mediate stress resilience and stress vulnerability. We hypothesize that prenatal stress has epigenetically regulated effects on health and disease of the nervous system from early development to old age. This review will discuss transgenerational epigenetic inheritance and the role of stress and epigenetic mechanisms in fetal programming and brain development. Furthermore, we will highlight the role of prenatal stress and associated epigenetic marks in influencing the risk of mental disorders such as schizophrenia, anxiety- and depression-related disorders, attention deficit hyperactivity disorder and autism later in life.

Transgenerational epigenetic inheritance: significance and definitions

Epigenetics is the study of heritable changes in the gene expression profile of a cell that are not caused by changes in the nucleotide sequence of the DNA. There are four main epigenetic

components: DNA methylation, histone modification and chromatin remodeling as well as non-coding RNA-mediated modifications. Out of these, DNA methylation is the most widely investigated and best understood component, followed by a recent increase in interest to determine the role of small non-coding RNAs, such as microRNAs (miRNAs), in health and disease.

Transgenerational epigenetic inheritance can be broadly defined as the transfer of epigenetic information across generations. It has been textbook knowledge for decades that the DNA sequence is the only heritable component that defines a phenotype; however, strong evidence now suggests a pivotal involvement of epigenetic components in determining phenotypic inheritance (Bruxner & Whitelaw 2008). Despite a recent surge of interest in transgenerational epigenetic responses, the nature of these heritable effects remains controversial (Kovalchuk 2012). A considerable variety of existing definitions of epigenetics itself as well as the definition of transgenerational epigenetic changes makes it difficult to provide a comprehensive summary of current advances in the field. The current review will use the terminology proposed by Crews (2008) and Kovalchuk (2012) that are described below.

Crews (2008) suggested the classification of epigenetic changes into heritable and context-dependent modifications. Heritable (meiotic, across generation) epigenetic modifications are those that occur in the germline, whereas context-dependent (mitotic, within generation) changes occur in somatic cells and persist only for the duration of the lifetime of an organism (Crews 2008). It was aptly noted by Crews (2008) and Skinner (2008) that there are two critical criteria required to demonstrate the germline-dependent epigenetic inheritance of the observed alteration in the phenotype. The first criterion is the exposure of one generation to an (usually adverse) event that was never again repeated in subsequent generations and the number of generations that passed since that exposure (Crews 2008, Skinner 2008). Accordingly, the second criterion is a minimum of two generations in case of male exposure or three generations in case of female exposure to claim truly transgenerational inheritance (Skinner 2008). Thus, it is worth

noting that intergenerational changes seen in the F1 and F2 generations are not necessarily heritable, since some of the modifications found in the subsequent generations may be attributed to the direct effects of germline exposure.

At the same time, as noted by Kovalchuk (2012), transgenerational variations are not always epigenetic in nature. For example, the accumulation of proteins and metabolites in the cytoplasm of maternal gametes in response to stress may have a profound effect on the development and cellular functions of the organism and the ultimate phenotype of the offspring. These transgenerational effects, however, are hardly epigenetic in nature (Kovalchuk 2012). In the current review we will use the definition proposed by Kovalchuk (2012), noting that transgenerational epigenetic inheritance is a transfer of the phenotypic appearance as a result of the transfer of epigenetic marks that are solely responsible for the observed phenotypic changes.

Some examples of transgenerational epigenetic inheritance have been described in different organisms, including plants, worms, fruit flies, and rodents. For example, in plants transgenerational epigenetic inheritance was demonstrated for flower symmetry in *Linaria vulgaris* (Cubas et al 1999), for flower colour in maize (Brink 1956, Woodhouse et al 2006), and in stress tolerance and changes in genome stability in *Arabidopsis* and tobacco exposed to various abiotic and biotic stressors (Boyko et al 2010, Boyko et al 2007, Kathiria et al 2010, Kovalchuk & Kovalchuk 2003, Luna et al 2012, Rasmann et al 2012, Slaughter et al 2012). Furthermore, in the nematode *Caenorhabditis elegans*, transgenerational epigenetic effects were described with regard to sterility (Katz et al 2009) as well as longevity (Greer et al 2011), and in *Drosophila melanogaster* with regard to eye colour (Cavalli & Paro 1998, Cavalli & Paro 1999) as well as heat stress response (Seong et al 2011).

In mammals, evidence of transgenerational epigenetic changes was reported for the coat colour in mice (Blewitt et al 2006, Morgan et al 1999), and in rats with regard to the effects of endocrine disruptors during pregnancy on spermatogenesis and subfertility in males (Anway et al 2005, Skinner et al 2013a). Effects of chemical endocrine disruptors on F1-F3 generations were

also demonstrated in female rats, where vinclozolin exposure during gonadal sex determination led to the transgenerational increase in pregnancy abnormalities and female adult onset of diseases (Nilsson et al 2008). However, these examples of transgenerational epigenetic effects are just the tip of the iceberg and we do not have a comprehensive understanding of the mechanisms of this heritable phenomenon yet. Data on humans are very limited at this time. Only a few epidemiological studies highlighted the possibility of transgenerational epigenetic changes, including the Överkalix study (Kaati et al 2002) and the Dutch Famine Cohort studies (Heijmans et al 2008, Kaati et al 2002). The above mentioned examples of transgenerational epigenetic changes and inheritance will be discussed in more detail below.

Epigenetic reprogramming during mammalian embryonic development

Most of the current knowledge about epigenetic reprogramming during embryonic development in mammals stems from work using mice (Dean et al 2001). In this context, epigenetic reprogramming refers to the erasure of DNA methylation marks and histone modifications (Guibert et al 2012, Hajkova et al 2008, Hajkova et al 2002, Popp et al 2010, Yamazaki et al 2003). Mouse studies revealed at least two global genome-wide epigenetic reprogramming events during mammalian development [reviewed in (Migicovsky & Kovalchuk 2011, Seisenberger et al 2013)]. The first event occurs between embryonic day E7.25 and E13.5 in mouse primordial germ cells (PGCs) after they reach the embryonic gonads (Ginsburg et al 1990, Hemberger et al 2009, Seisenberger et al 2012, Seki et al 2005, Seki et al 2007, Surani 1998). The recent study by Seisenberger et al. (2012) identified two specific phases of demethylation in the mouse PGCs: at first global demethylation occurs early during migration of PGCs, when methylation of specific regions is actively maintained, whereas the second demethylation event occurs upon entry of PGCs into the genital ridges (Seisenberger et al 2012). The following wave of remethylation is sex-specific. It begins on E14.5 in prospermatogonia in male germ cells, and after birth in growing oocytes in females (Davis et al 2000, Hajkova et al 2008, Li et al 2004, Ueda et al 2000). The second reprogramming event starts in the zygote

immediately after fertilization and extends to the morula stage of preimplantation development in the early embryo (Mayer et al 2000, Oswald et al 2000, Smith et al 2012). Both, germ cells and zygote, require epigenetic reprogramming that ensures erasure of DNA methylation and histone modifications to restore their totipotency (Surani et al 2007). Around the implantation period both maternal and paternal genomes undergo a wave of *de novo* methylation (Borgel et al 2010, Howlett & Reik 1991, Monk et al 1987, Sanford et al 1987). Interestingly, mouse mutants with inactivated *de novo* methyltransferases Dnmt3a and Dnmt3b die at postimplantation stages or early after birth (Okano et al 1999). This observation highlights that appropriate functioning of DNA methylation mechanisms is vitally important for proper mammalian development, particularly during early postimplantation embryonic stages.

Some epigenetic marks, however, may escape the reprogramming process, including imprinted genes [reviewed in (Bartolomei 2009)] and some regulatory elements (Borgel et al 2010, Hackett et al 2013, Smallwood et al 2011), resulting in transgenerational epigenetic inheritance and novel phenotypic traits. For example, work by Hackett et al. (2013) suggested that transgenerational epigenetic inheritance may result from the ability of rare regulatory elements (identified in the study) to escape the DNA demethylation in primordial germ cells. In this study the authors identified 11 CpG islands that escaped 5mC reprogramming in E13.5 PGCs (Hackett et al 2013). The whole-genome bisulfite sequencing of the mouse PGCs revealed that 4,730 loci escaped demethylation. Most of the loci that escaped demethylation were associated with repeats and corresponded predominantly to intracisternal-A-particles or telomeric regions (Hackett et al 2013). Based on these findings, the authors suggest that by evading both reprogramming events during embryonic development these 5mC epialleles may be inherited over multiple generations (Hackett et al 2013), but their potential functional role still needs to be investigated.

Transgenerational epigenetic changes in mammals

Despite the occurrence of genome-wide epigenetic reprogramming during both gametogenesis and embryogenesis in mammals, several incidents of epigenetic inheritance were

shown for a small number of genes in mice (Herman et al 2003, Morgan et al 1999, Rakyan et al 2003, Rassoulzadegan et al 2006). One of the strongest pieces of evidence of transgenerational epigenetic inheritance was demonstrated in a classic study using agouti mice (Morgan et al 1999). Other well established experimental work using animal models dealt with the transgenerational effects of endocrine disruptors (Anway et al 2005, Anway et al 2006a, Anway et al 2006b, Anway & Skinner 2008, Crews et al 2007) and stress (Franklin et al 2010). These studies and related evidence derived from human cohorts will be discussed in the following sections.

Metastable epialleles in mice

Using an agouti mouse model, Morgan and colleagues provided a classic example of transgenerational epigenetic inheritance in mammals (Morgan et al 1999, Rakyan et al 2003). In their studies the authors examined the metastable epialleles among genetically identical mice. Metastable epialleles are alleles that may be differentially expressed in genetically identical individuals due to the different epigenetic state of the loci (Rakyan et al 2002). Examples of such alleles include the *agouti viable yellow* (A^{vy}) and *axin fused* ($Axin^{Fu}$). Both epialleles contain an intracisternal A particle (IAP) transposable element that regulates the expression of linked genes via changes in the methylation status of a cryptic promoter in the IAP long terminal repeat (Youngson & Whitelaw 2008). A^{vy} alleles are caused by the insertion of an IAP element 100 kb upstream of agouti coding sequences (Duhl et al 1994). Mutations at the agouti gene are also associated with adult-onset obesity, diabetes, and tumorigenesis (Miltenberger et al 1997, Morgan et al 1999). The level of CpG methylation in the A^{vy} IAP promoter region correlates inversely with the ectopic expression of agouti protein, resulting in considerable individual variation in the coat colour of isogenic A^{vy}/a mice (Morgan et al 1999). The variation in the fur color ranges from dark-brown pseudoagouti (methylated) to yellow (unmethylated) in A^{vy} isogenic mice. Interestingly, IAPs are largely resistant to DNA methylation reprogramming during early embryogenesis, thus enabling heritable epimutations of neighbouring genes (Lane et al 2003). Similarly, in $Axin^{Fu}$ isogenic mice the phenotypic variability correlates with the differential methylation state of an

IAP sequence at the *Axin* locus, where hypomethylation is associated with an aberrant "kinky tail" phenotype (Rakyan et al 2003).

"Paramutation-like" effects in mice and the role of sperm RNA in transgenerational epigenetic inheritance

Paramutation is a pattern of inheritance that contradicts the classical Mendelian laws and results in heritable changes in gene expression that occur upon allelic interactions. This phenomenon is well documented in plant research (Bateson & Pellew 1915, Brink 1958, Meyer et al 1993, Sidorenko & Peterson 2001). Intriguingly, cases of paramutation-like effects have also been reported in mouse studies (Cuzin & Rassoulzadegan 2010, Grandjean et al 2009, Herman et al 2003, Kiani et al 2013, Rassoulzadegan et al 2006, Wagner et al 2008). The first insights into this phenomenon in mice were provided in a study by Rassoulzadegan et al. (2006), in which the authors observed an interallelic cross-talk at the *Kit* gene. *Kit* mouse mutants created by lacZ cassette insertion display obliterated production of the KIT protein. Null mutants of the Kit gene are lethal in the homozygous state and die shortly after birth, whereas heterozygous mice are characterized by white feet and a white tail tip (Rassoulzadegan et al 2006). Interestingly, the progeny of intercrosses between heterozygotes or crosses with wild-type animals showed a reduced number of offspring with wild-type phenotype than was expected according to Mendelian inheritance. Closer molecular analysis revealed that some mice that displayed the mutant phenotype were in fact genetically wild-type at the *Kit* locus, thus displaying a paramutation-like effect (Rassoulzadegan et al 2006). Their phenotype was termed *Kit**.

Interestingly, *Kit** mice transmitted their mutant-type phenotype to the progeny (Rassoulzadegan et al 2006). The number of *Kit* transcripts in these animals was reduced on a posttranscriptional level, however, the rate of transcription was elevated in both somatic and germ cells. Microinjections of RNA from somatic and germ cells of heterozygotic mice to the wild-type one-cell embryo increased the frequency of the heritable white tail phenotype, whereas wild-type RNA injections of the control embryos showed the mutant phenotype at a low efficacy with

inefficient transmission to the progeny (Rassoulzadegan et al 2006). Moreover, the injection of two miRNAs (miR-221 and -222) that are partially complementary to the *Kit* RNA was also able to induce the paramutated state in contrast to injection of the variety of other miRNAs tested (Cuzin et al 2008, Rassoulzadegan et al 2006).

In a subsequent study by the Rassoulzadegan laboratory, the authors were able to demonstrate another paramutation-like effect by injecting fertilized mouse eggs with RNAs targeting Cdk9 (a key regulator of cardiac growth), inducing cardiac hypertrophy (Wagner et al 2008). Microinjections of miR-1 or fragments of the Cdk9 coding regions induced elevated expression of homologous RNA. Interestingly, cardiac hypertrophy in miR-1 injected mice was not caused by the down-regulation of the miRNA, since no significant changes in the miR-1 expression were observed in embryonic hearts. The observed paramutation-like effect was heritable and correlated with miR-1 presence in the sperm nucleus (Wagner et al 2008). Cardiac hypertrophy was inherited in crosses of either male or female miR-1-injected parents with normal partners. The crosses generated progenies with enlarged hearts in about 90% of the cases across at least three generations (Wagner et al 2008). Thus, the above mentioned experiments highlight the possibility that sperm RNA is a candidate signal responsible for paternal inheritance.

Further research provided evidence supporting the role of sperm RNA, including miRNAs as a transgenerational signal (Grandjean et al 2009). This time researchers from the Rassoulzadegan group injected miR-124 into fertilized eggs that increased the size of the mouse pups born by 30%. It is important to note that miR-124 is a brain-specific miRNA that is important for central nervous system development (Cao et al 2007, Makeyev et al 2007, Visvanathan et al 2007). The observed "giant" phenotype was maintained into adulthood and was transmitted to the second generation, whereas the F3 progeny of crosses between miR-124-injected animals with wild-type partners displayed the normal average weight. Similarly to the previous studies, the paramutant phenotype was not associated with the altered miR-124 expression, since its copy numbers returned to the levels of the control animals shortly after

(Grandjean et al 2009). Thus, the authors suggested that it is more likely that the induced phenotype is a result of initial exposure of the fertilized eggs to the RNA with sequence homology to the targeted transcript rather than the result of the permanent alteration in miRNA expression (Grandjean et al 2009). Indeed, the authors found that transcripts from the loci with sequence similarities to miR-124, such as *Sox9, LamC1*, and *Acaa2*, were significantly up-regulated in embryos developing from miR-124-injected eggs.

Grandjean et al. (2009) also demonstrated that miR-124 injections into eggs induced permanent heritable changes to the chromatin structure at the *Sox9* promoter region. On day E6.5 of development, miR-124 injection into fertilized eggs caused an increase in the methylated forms of histone H3 (H3K9me2 and me3) (Grandjean et al 2009). Finally, a recent study by Kiani et al. (2013) demonstrated that the expression of the Dnmt2 RNA methyltransferase is required for the establishment and hereditary maintenance of paramutation-like effects at both *Sox9* and *Kit* loci (Kiani et al 2013). The authors reported that *Sox9* paramutation was not established in Dnmt2$^{-/-}$ embryos. Similarly, the injection of the RNA from the brains and testes of Dnmt2-deficient *Kit* heterozygotes was not able to induce the paramutant *Kit* phenotype.

Implication of sperm RNA in transgenerational inheritance of stressful experiences

The role of sperm RNA in transgenerational inheritance was recently demonstrated in mice subjected to traumatic stress (Gapp et al 2014). Using a mouse model of unpredictable maternal separation combined with unpredictable maternal stress, Gapp et al. (2014) showed that traumatic stress in early life altered mouse miRNA expression in sperm, serum and brain, as well as behavioural and metabolic responses in the progeny. MiRNA and piRNA profiles of sperm from F1 males subjected to early life stress were altered. MiRNAs were also affected in serum, hippocampus and hypothalamus of stressed F1 and F2 animals, but not in F2 sperm (Gapp et al 2014). Interestingly, miRNA profiles were normal in the F3 generation. Microinjection of sperm RNAs from stressed males into fertilized wild-type oocytes produced similar behavioural and metabolic alterations in the progeny (Gapp et al 2014).

The important role of sperm miRNAs in HPA stress axis regulation was previously demonstrated by Rodgers et al. (2013). Six weeks of chronic stress exposure of male mice before breeding caused reduced HPA stress axis response in the offspring accompanied by the alteration of miRNA profiling in paternal sperm (Rodgers et al 2013). Another recent study examined transgenerational effects of stressful experiences (Dias & Ressler 2014). In this study the parental generation (F0) of male mice was subjected to odor fear conditioning before conception and subsequent F1 and F2 generations were investigated. The results showed that subsequently conceived generations displayed F0-like behavioral sensitivity toward the F0-conditioned odor. Moreover, bisulfite sequencing of olfactory receptor genes in the sperm DNA from conditioned F0 and naive F1 males showed CpG hypomethylation in the *Olfr151* gene. Thus, these studies highlight the importance of stress and ancestral experience before conception for the well-being of the future offspring, as well as the intriguing role of sperm RNA in transgenerational inheritance.

Transgenerational non-genetic effects

The transmission of a specific phenotype to subsequent generations has been studied since the early 1980s. A line of research by Kahn (1970, 1982) began with a study in a mouse model, which demonstrated that restricted air circulation during adulthood prior to mating causes altered blood hemoglobin concentrations in the female but not in the male offspring (Kahn 1970). A follow-up study reported transgenerational transmission of changes in hemoglobin concentration in response to an adverse prenatal environment (Kahn 1982). Gestational females were kept either in poorly ventilated cages or provided with a diluted yeast RNA in their drinking water (Kahn 1982). Experimental and control lines were then studied for three successive generations. Their results showed that hemoglobin concentration were significantly higher in F1 generation experimental animals than in control animals, whereas F2 and F3 progenies of experimental animals exhibited decreased levels of hemoglobin in comparison to controls (Kahn 1982).

Further evidence of non-genetic transgenerational effects derived from a study by Huck et al. in 1987. Using a hamster model the authors investigated the long-lasting consequences of food restriction during the first 50 days of life on sex ratios and offspring growth trajectories in subsequent generations (Huck et al 1987). Intriguingly, they showed that food-restriction in F1 females resulted in significantly restricted growth and smaller litters in the F3 generation by the age of 5-25 days compared to control litters fed an *ad libitum* diet. Moreover, the sex ratio in the F3 generation offspring that descended from food-restricted females was altered, with a significantly lower percentage of males per litter. In summary, these reports of inherited phenotypic traits suggest the existence of transgenerational non-genetic effects that may be transmitted via the gametes.

Transgenerational effects of endocrine disruptors

Exceptional examples of transgenerational epigenetic inheritance have been reported in studies involving endocrine disruptors in rats. It is widely accepted that endocrine-disrupting chemicals can alter molecular epigenetic regulation, including DNA methylation and histone modifications. As defined in a Statement of Principles by *The Endocrine Society*, an endocrine-disrupting chemical (EDC) is "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Zoeller et al 2012). There is a substantial body of evidence of transgenerational epigenetic effects induced by EDCs, such as vinclozolin, diethylstilbesterol, bisphenol A, and polychlorinated biphenyls, in mammals (Walker & Gore 2011). Compelling examples of disturbing actions of EDCs on future generations via epigenetic perturbations are reviewed in Walker and Gore (2011), and the potential evolutional impact is discussed by Crews and Gore (2012) (Crews & Gore 2012, Walker & Gore 2011).

The laboratory of Michael Skinner has extensively studied the transgenerational epigenetic effects in response to prenatal exposure to different endocrine disruptors, such as fungicides, pesticides, and other environmental toxins (Anway et al 2005, Anway et al 2006a, Anway et al 2006b, Anway & Skinner 2008, Crews et al 2007). The authors found that prenatal

exposure to vinclozolin, a common dicarboximide fungicide, produced wide-ranging adverse health effects in both male and female offspring. In males, for example, it led to a number of adult disorders, including reproductive abnormalities, cancer, prostate and kidney diseases (Anway et al 2006a). Among effects on female offspring, the authors found an association between prenatal exposure to endocrine disruptors and the onset of ovarian diseases later in life across multiple generations (Nilsson et al 2012). In these studies, the F0 generation of gestating female rats was exposed to different EDCs during embryonic gonadal sex determination. Ovarian diseases were then assessed in the F1 and F3 progeny (Nilsson et al 2012). Interestingly, both the F1 and F3 generations of offspring displayed ovarian disease phenotypes, including an increase in cysts resembling human polycystic ovarian disease (PCO) and a decrease in the ovarian primordial follicle pool size resembling primary ovarian insufficiency (POI) (Nilsson et al 2012). Moreover, this group of researchers reported differences in mRNA expression and DNA methylation in the granulosa cells in F3 generation progeny of EDC-exposed animals as compared to controls (Nilsson et al 2012). Since F3 generation animals were not exposed to EDC, changes in their epigenome likely represent a truly transgenerational effect.

Transgenerational effects of stress

Stress may be able to induce phenotypic epigenetic changes across multiple generations similar to those seen in environmental toxicant studies (Skinner, 2014). The laboratory of Mansuy and colleagues studied the effects of chronic and unpredictable maternal separation in early life on the F2 and F3 offspring (Franklin et al 2010). F1 offspring were exposed to chronic and unpredictable maternal separation from postnatal day 1 to 14. Subsequently, adult F1 male offspring (both stressed and control) were bred with wild-type females to produce the F2 generation. Adult F2 offspring (both stressed and control lineage) were bred with wild-type females to produce the F3 generation. Therefore the actual stress exposure occurred in the F1 generation only. This experimental design allowed the authors to assess the impact of the stress exposure during early life on the subsequent F2 and F3 generation offspring. The authors

observed a depressive-like phenotype in the F1 male offspring that were subjected to stress in early life in comparison to controls, but not in F1 females. Interestingly, similar behavioural traits were observed in F2 female but not F2 male offspring. Strikingly, F3 male offspring expressed similar symptoms of depression as F1 males. These results suggest that depressive-like symptoms can be transmitted across generations in a complex and sex-specific mode (Franklin et al 2010). Moreover, this study reported that chronic and unpredictable maternal separation alters the DNA methylation profile in the promoter of several candidate genes in the germline of stressed males, thus propagating to the F3 generation. Some of these changes were also present in the brains of F2 offspring (Franklin et al 2010).

In addition, our recent study demonstrated that prenatal stress increases the risk of shortened gestational length that is present across multiple generations of rats (Yao et al 2014). Pregnant dams of the parental generation (F0) were exposed to stress from gestational days 12 to 18. Their pregnant daughters (F1) and grand-daughters (F2) were either stressed or remained non-stressed. Both of the stress-treated lineages (stress only in the F0 generation or across all generations) showed gradually reduced gestational length, maternal weight gain and maternal behavioural activity across subsequent generations. Moreover, a family history of stress impaired offspring sensorimotor development with the most severe impairments occurring in the F3 generation. The behavioural and physiological changes in these animals were accompanied by altered miRNA expression in the brain and uterus of F2 mothers (Yao et al 2014). Altered miRNA expression included the miR-200 family, such as upregulation of miR-200b and downregulation of miR-429 (Yao et al., 2014), both of which were suggested to modulate gestational length through interaction with their gene targets (Renthal et al., 2010). The findings by Yao et al. (2014) suggest that recognizable epigenetic signatures of preterm birth, behaviour and physiology are programmed through the maternal lineage.

Transgenerational effects of diet in mice

Another potentially adverse experience with associated epigenetic changes involves the experimental variation of dietary regimens. A series of classic studies have demonstrated that dietary components can modulate DNA methylation patterns related to obesity. The agouti viable yellow mouse model allows researchers to investigate the impact of environmental and dietary influences on the fetal epigenome (Dolinoy 2008). It has been shown that diet-induced hypermethylation during development can rescue an obese phenotype in agouti mice (Cooney et al 2002, Waterland et al 2008, Wolff et al 1998). To verify transgenerational inheritance of induced epigenetic variation, Waterland et al. (2007) examined three generations offspring that were weaned on either a methyl-supplemented or a control diet (Waterland et al 2007). Their results showed that in the methyl-donors-supplemented groups coat colours were darker than in controls, however, no cumulative effects of supplementation across successive generations were observed. Thus, the authors concluded that diet-induced hypermethylation at agouti viable yellow is not inherited transgenerationally through the female germ-line (Waterland et al 2007).

Interesting evidence of transgenerational inheritance in mice was demonstrated by the Bale laboratory (Dunn & Bale 2009, Dunn & Bale 2011). In these studies the authors reported that maternal high-fat diet exposure led to increased body size and reduced insulin sensitivity that persisted to the F2 generation via both maternal and paternal lineages (Dunn & Bale 2009). To verify a robust heritable transgenerational epigenetic effect, Dunn and Bale analysed the F3 generation (Dunn & Bale 2011). Interestingly, their results revealed no alterations in insulin sensitivity in the F3 offspring, and transgenerational inheritance of the increased body size phenotype was observed in females only. Intriguingly, these effects were transmitted via the paternal lineage, suggesting involvement of genomic imprinting (Dunn & Bale 2011). Analysis of the expression of imprinted genes, involved in growth, in the livers of F3 females derived from the paternal lineage revealed a trend towards greater volatility (greater than 50% deviation from baseline in either direction) of expression than those of the maternal lineage (Dunn & Bale 2011).

These findings provide evidence of sex-specific transgenerational programming of phenotypic traits, such as growth and body size, being likely due to epigenetic modifications (e.g. a stable epigenetic mark in the paternal gametes) rather than genetic changes. The potential role of imprinted genes in this sex-specific epigenetic programming requires further investigation.

Transgenerational epigenetic inheritance in humans

Despite the intriguing examples of transgenerational epigenetic inheritance in rodents, the idea that epigenetic marks can be inherited in humans remains controversial. To date several studies reported heritable germline epimutations in humans (Buiting et al 2003, Chan et al 2006, Hitchins et al 2007, Suter et al 2004). However, it still needs to be verified whether the described cases of epimutations are causally related to transgenerational inheritance, since heritable germline epimutations differ from transgenerational epigenetic inheritance (Chong et al 2007, Horsthemke 2007). Heritable epimutations (aberrant methylation marks observed in more than one generation) may not necessarily be the result of transgenerational epigenetic inheritance, since atypical methylated states can be erased during gametogenesis and then re-established in the zygote as a result of a change in the DNA sequence (Chong et al 2007, Horsthemke 2007). Work from two epidemiological cohorts, described below, suggests, however, the intriguing possibility of transgenerational epigenetic inheritance.

The Dutch Famine Cohort Study

The Dutch Famine or "Hunger Winter" took place in 1944-1945 in a part of the Netherlands that was occupied by Germany. As a result of the German blockade access to food supplies in the occupied part of the country was extremely limited and millions of people were unable to maintain adequate nutrition during unusually harsh winter conditions. Daily adult rations ranged from 580 to 1,000 kilocalories per day. The Dutch Famine Birth Cohort was created to include affected mothers and fathers and their children to form a systematic study of the effects of perinatal undernutrition. This cohort has been extensively studied and multiple consequences of famine exposure were reported in perinatal epidemiology. For example, it was

shown that children of pregnant women exposed to the famine during pregnancy were more susceptible to coronary heart disease, diabetes, obesity, microalbuminuria and accelerated cognitive aging (Painter et al 2006, Painter et al 2005a, Painter et al 2005b, Ravelli et al 1999, Roseboom et al 2001). The effects of prenatal undernutrition on adult health, however, significantly depended on the timing of exposure during gestation (Roseboom et al. 2001). The mechanisms of the observed phenotypic changes are yet to be clarified, however, it is likely that epigenetic regulation is involved. Heijmans et al. (2008) investigated the changes in the DNA methylation in the offspring of the mothers who were exposed to the Dutch Famine. Their results showed that individuals that were conceived during the Dutch Hunger Winter had less DNA methylation of the imprinted IGF2 gene compared with their unexposed, same-sex siblings 60 years later (Heijmans et al 2008). No such changes in DNA methylation were found in individuals who were exposed while being *in utero* later during gestational periods. This study suggests that potentially heritable DNA imprinting caused by periconceptional exposure to undernutrition has potential consequences for health in late adulthood. Another human study that is discussed below highlights the possibility that even grandparents' exposure to an adverse environment and nutritional resources may influence their grandchildren through epigenetic modifications.

The Överkalix Cohort Study

Another unique historical cohort is investigated by the Överkalix studies. Överkalix is a small isolated municipality in Sweden that has an extensive set of historical records about its population, which includes the information on food availability during different years, land ownership, causes of death, etc. Taking advantage of the Överkalix historical records on harvests and food prices, Bygren and colleagues investigated whether food availability during a child's slow growth period (SGP) can influence the descendants' longevity as well as risk of death from cardiovascular disease and diabetes (Bygren et al 2001, Kaati et al 2002). Their results suggest that paternal grandfathers' overeating during the SPG increases the likelihood of grandchildren's death from diabetes by four-fold (Kaati et al 2002). Interestingly, if a father experienced poor

food availability or a famine during his SGP then his son was protected against cardiovascular death (Kaati et al 2002).

This group of researchers also investigated an association between longevity and food availability during the grandparents' SGP (Bygren et al 2001). In their study data from children of different ages from the Överkalix cohort were analyzed, including the ages of 0-2 years for girls and boys, 3-7 years for girls, 3-8 years for boys, 11-15 years for girls, and 13-16 years for boys. Notably, food availability during the prepubertal period only appeared to have a profound effect on the grandchildren longevity, skipping the generation of children (Bygren et al. 2001). The survival of the grandchildren was shortened by 16.5 ± 6 years if there was a surfeit of food in the environment during their grandfathers' SGP (when he was 9-12 years old). Good and poor food availability had the opposite effects on grandchild survival in contrast to moderate food supply that had no effect. There were no significant effects of food availability during parental or grandmothers' SGPs or other age periods (Bygren et al 2001). Despite this convincing evidence from human cohorts, the mechanisms and stability of epigenetic inheritance of complex phenotypes in experimental studies is still being discussed (Berger 2012).

Fetal programming

The concept of fetal origins of adult disease or fetal programming was developed by David Barker and colleagues based on the observation that a low birth weight increases the risks of cardiovascular disease and type 2 diabetes in later life (Barker et al 1993b). This concept proposes that fetal adaptations to the intrauterine and maternal environments shape the structure and function of organs, leading to permanent physiological alterations in adulthood (Swanson et al 2009). Therefore, this approach suggests that the predisposition to diseases in adulthood is "programmed" *in utero*. Barker's early observation about the role of the fetal environment in the future development of cardiovascular disease has since been extended to other diseases, including conditions affecting the brain, such as psychiatric disorders. For example, evidence from

epidemiological studies related maternal psychological stress caused by bereavement, unwanted pregnancies, military invasions and natural disasters to an elevated risk for the offspring to develop schizophrenia later in life (Huttunen & Niskanen 1978, Khashan et al 2008, Khashan et al 2011a, Kinney et al 1999b, Myhrman et al 1996b, Selten et al 1999a, van Os & Selten 1998b). Yet the molecular mechanisms of fetal programming remain poorly understood. Over the past two decades, epigenetic studies have become a promising field in revealing the mystery of fetal programming. Many of epigenetic differences arise during development and remain stable throughout life. For example, it has been shown that environmental influences during early postnatal life can cause DNA methylation changes in the promoter regions of glucocorticoid receptors, one of the two receptor types for glucocorticoids, the main stress hormones, in the brain (McGowan et al 2009b, Weaver et al 2004). Accordingly, maternal stress during pregnancy may critically influence the density of glucocorticoid receptors in areas of the fetal brain, particularly the hippocampus, and permanently alter the sensitivity to stress throughout life (McGowan et al 2009b, Weaver et al 2004) arguably through epigenetic mechanisms involving DNA methylation and miRNA expression (Babenko et al 2012a, Weaver et al 2004).

It is important to note that these studies present the challenge of dissociating the environmental effects *per se* from a potential genetic predisposition of an individual to certain diseases. For example, gene-environment interactions that occur when adverse environmental factors synergistically interact with a certain genetic predisposition, may in fact account for a significant fraction of psychiatric disease cases (Caspi & Moffitt 2006). According to the hypothesis of genetic moderation, differences between individuals that derive from differences in the DNA sequence promote individual differences in the response to environmental conditions (Caspi & Moffitt 2006). This should be taken into consideration when studying fetal or early-life programming aspects, since there is substantial evidence for the role of gene-environment interactions in determining stress sensitivity in both human (Caspi et al 2002, Caspi et al 2003,

Kim-Cohen et al 2006) and animal studies (Ayhan et al 2009, Barr et al 2004, Oliver & Davies 2009).

MicroRNAs (miRNA) are important epigenetic regulators of brain development

MiRNAs are small non-coding RNAs approximately 22 nucleotides long that function as regulators of gene expression at the transcriptional and post-transcriptional levels. Despite the fact that miRNAs were first discovered in the early nineties, their role in the nervous system only recently started to be appreciated. During the past decade, growing evidence demonstrated the essential role of miRNAs in neuronal development and neuronal function (Krichevsky et al 2003, Schratt et al 2006) (reviewed in (Sun et al 2013)). For example, microRNA expression patterns were studied during brain development in specific cell populations, including neurons (Kim et al 2004, Kye et al 2007), oligodendrocytes (Lau et al 2008, Letzen et al 2010), astrocytes (Smirnova et al 2005), and microglia (Ponomarev et al 2011). In addition to these studies performed in rodents and cell cultures, a recent report by Moreau et al. (2013) measured miRNA expression in the developing human brain. Their expression profiling revealed distinct temporal expression patterns of miRNAs in post-mortem brain tissues representing gestational ages 12-24 weeks, as well as early postnatal and adult time points (Moreau et al 2013).

Despite the recent interest in neuronal miRNA expression the understanding of exact miRNA functions in the healthy and injured nervous system is far from being complete. The fact that single microRNA can modulate the expression of multiple genes adds an additional level of complexity to the unambiguous identification of the role of a specific miRNA in the brain. The current agreement in the literature suggests the function of miRNA in fine-tuning gene expression. Compared to transcriptional repressors, miRNAs can influence target protein levels more rapidly at the post-transcriptional level (Tsang et al 2007). Interestingly, it was demonstrated that miRNAs control *de novo* DNA methylation in mouse embryonic stem cells through regulation of transcriptional repressors (Sinkkonen et al 2008). On the other hand, it was also shown that post-mitotic neural development and dendritic morphogenesis is regulated by

miRNAs via switching of chromatin-remodeling complexes (Yoo et al 2009). Thus, miRNAs may be involved in different levels of epigenetic regulation.

miRNAs in placenta

The placenta is a vital transient organ that connects the developing fetus to the maternal uterine wall. It participates in the exchange of gases, nutrients, hormones and waste between embryonic and maternal environments, and it serves important endocrine and protective functions. Pathological processes in the placenta are frequently associated with complications during pregnancy, including preterm birth, preeclampsia, and fetal growth restriction (Faye-Petersen 2008, Huppertz 2011) (Figure 1.1). The role of miRNAs in regulating placental-specific gene expression in normal and pathological conditions is not clear. Increasing evidence suggests that miRNAs are important regulators of placental development, however, their role in the placental stress response have not been sufficiently studied. Abnormal miRNA expression in placenta has been reported in compromised pregnancies (Fu et al 2013, Mouillet et al 2011). Previous studies particularly focused on preeclampsia, which is associated with specific miRNA signatures, such as overexpression of miR-34a in preeclamptic placentas (Doridot et al 2013). Studies like these highlight the potential of placental miRNAs as biomarkers of pregnancy outcomes and offspring development.

DNA methylation in the embryonic brain

DNA methylation is one of the best characterized epigenetic modifications (recently reviewed by (Smith & Meissner 2013) and (Wu & Zhang 2010)). In mammals, DNA methylation occurs predominantly at CpG dinucleotides and involves a covalent addition of a methyl group to the 5-position of cytosines by enzymes called DNA methyltransferases (DNMTs). Some members of the DNMT family serve as *de novo* methyltransferases, for example DNMT3A and DNMT3B (Okano et al 1999), while DNMT1 ensures that an established DNA methylation pattern is maintained during cell divisions (Bestor 2000). The process of establishing and maintaining a DNA methylation pattern is complex and involves the interaction between DNMTs and methyl-

CpG binding proteins (MBDs). MBDs are the proteins that read and interpret the information about methylation patterns (Newell-Price et al 2000, Wade 2001).

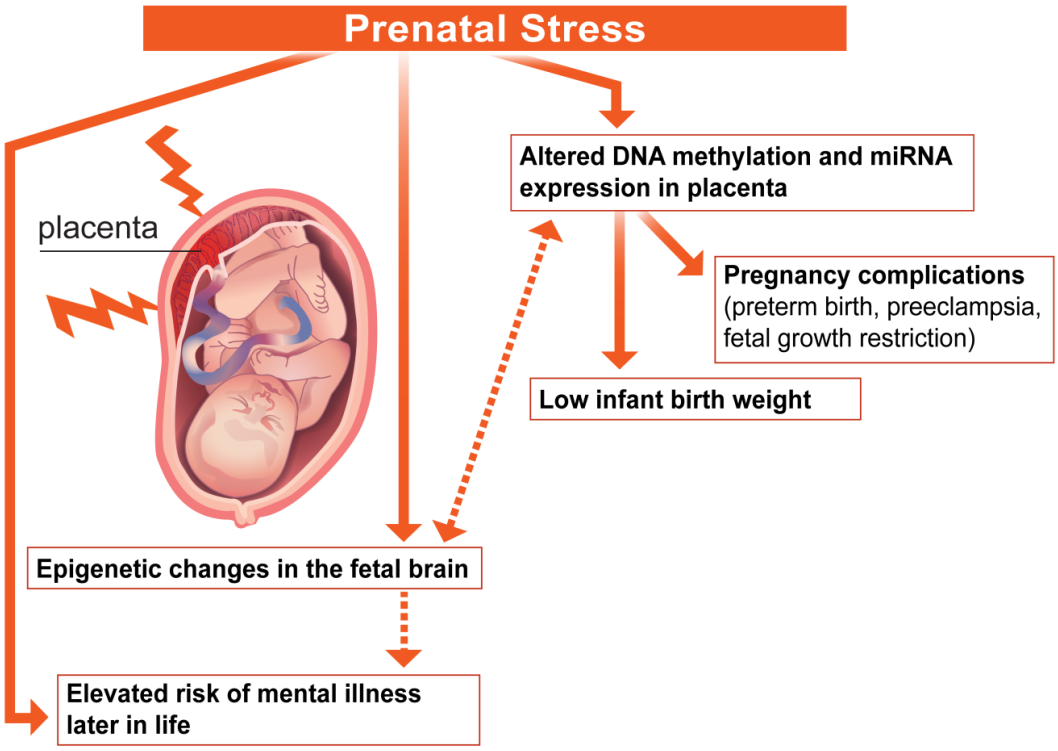


Figure 1.1. Association between prenatal stress and mental health (graphic support, Amanda McRoberts). Proper functioning of miRNA and DNA methylation mechanisms are required for normal placental and brain development. Human studies showed that alteration in these epigenetic mechanisms are associated with pregnancy complications and low infant birth weight. Animal studies demonstrated that stress can induce epigenetic changes in placenta and brain. Whether stress-induced epigenetic changes in the placenta and fetal brain are causally linked to the risk of mental illness later in life is yet to be verified.

DNA methylation plays a critical role during mammalian development (Li et al 1992). It has a variety of functions and is required for several processes, such as silencing of transposable elements and pericentromeric repeats to ensure genome integrity (Chen et al 2004, Kaneko-Ishino & Ishino 2010, Walsh et al 1998, Xu et al 1999), inactivation of X-chromosomes (Lock et al 1987, Mohandas et al 1981, Sado et al 2004) and genomic imprinting (Feil & Khosla 1999, Reik et al 1987).

It was recently discovered that in addition to the classical DNA methylation variant (5-mC), the family of TET proteins (cytosine oxygenase enzymes) is responsible for oxidizing 5-methylcytosine into 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), thus giving rise to other DNA methylation variants (Cadet & Wagner 2013, Williams et al 2012). The 5-hmC variant is now considered as a sixth DNA base and has unique properties in the brain (Nestor et al 2012). It was shown by Nestor et al. (2012) that the levels of 5-hmC in the brain are drastically higher than those of other tissues. The biological function of 5-hmC remains unknown, however, some evidence suggests its role as an intermediate agent in the DNA demethylation processes, as well as a possible role in transcriptional regulation through modifying levels of chromatin accessibility to the transcription machinery or to the MBDs (Nestor et al 2012, Song et al 2011, Tahiliani et al 2009, Valinluck et al 2004). Cytosine hydroxymethylation is present in the germline and at fertilization, suggesting a compelling role in transgenerational epigenetic inheritance (Smith & Meissner 2013).

A critical role of DNA methylation in embryonic development was demonstrated in experiments using mutant mice lacking DNA methyltransferases. DNMT1 mutant mice fail to develop beyond the stage characteristic of normal E9.5 embryos and die prior to E11 (Li et al 1992). To overcome early embryonic lethality Fan et al. (2001) generated DNMT1 conditional mutants to study the particular role of this methyltransferase in the developing brain. Their approach allowed to investigate the function of DNMT1 either on E12 in the fetal neuroblasts, or after birth, in postmitotic neurons of postnatal mice (Fan et al 2001). Their results showed that mitotic neuroblasts are sensitive to Dnmt1 deletion that caused DNA hypomethylation in daughter cells. Mouse mutants possessing 95% hypomethylated cells in the brain died immediately after birth. Interestingly, postmitotic Dnmt1 deletion did not compromise cell survival during postnatal life and did not affect the levels of global DNA methylation in postmitotic neurons (Fan et al 2001).

Further genetic manipulation studies demonstrated that double knockout mice lacking both Dnmt1 and Dnmt3A in adult forebrain neurons have impaired synaptic plasticity, learning and memory deficits (Feng et al 2010, Hutnick et al 2009). Hutnick et al. (2009) produced conditional DNMT1 mouse mutants that carried about 90% of hypomethylated cortical and hippocampal cells in the dorsal forebrain starting on E13.5 onward. Those animals displayed severe cortical and hippocampal degeneration between E14.5 and three weeks postnatally (Hutnick et al 2009). A recent study by Zhang et al. (2013) showed that Tet1 is also required for adult neurogenesis and normal cognitive functions of the mouse hippocampus (Zhang et al 2013). Moreover, DNMT1 was recently implicated in retinal network formation during brain development (Rhee et al 2012). Mouse mutants lacking DNMT1 in the retina displayed signs of abnormal neuronal differentiation of photoreceptors as well as rapid cell death of several types of neurons in the postnatal retina (Rhee et al 2012).

Interestingly, in humans DNTMT1 mutations cause neurodegeneration in the form of hereditary sensory neuropathy with dementia and hearing loss (Klein et al 2011). Mutations in another player of the DNA methylation machinery, the methyl binding protein MeCP2, were also implicated in various neurodevelopmental disorders, including severe neonatal encephalopathy, X-linked mental retardation, autism, and Rett syndrome [(Goffin et al 2012); for more details see (Gonzales & LaSalle 2010)]. Despite recent insights that indicated a causal connection between epigenetic regulation and cognitive function, little is known about the underlying molecular mechanisms (Bender & Weber 2013).

To reach further conclusions about the role of DNA methylation, Lister et al. (2013) took a genome-wide mapping approach at a single-base resolution in both human and mouse frontal cortex throughout their lifespan (Lister et al 2013). The authors found that levels of DNA methylation in a non-CG context (mCH, where H=A, C or T) were negligible in the fetal cortex but occurred abundantly in the adult frontal cortex. In mice, rapid accumulation of DNA methylation in a non-SG context during early postnatal development was accompanied by the up-

regulation of DNMT3a, suggesting that this DNA methyltransferase may be responsible for non-CG methylation (Lister et al 2013). However, methylation at 5-hmC that is acquired in the cortex during postnatal development, occurred exclusively in the CG context. Their results also showed that of the total methylated fraction in adult human neuronal cortex, mCG accounted for ~47%, whereas non-CG context constitutes ~53%, with mCH regions being highly conserved between unrelated individuals (Lister et al 2013). These authors also found a small number of "mCH deserts", which represent megabase-sized regions in the adult cortex that do not accumulate mCH and 5-hmC (however, mCG is not depleted in these regions) (Lister et al 2013). Furthermore, in this study mCH deserts had lower chromatin accessibility and were enriched for large clusters of genes involved in immunity and receptors for sensory neuron function. Cell type-specific variation in methylation patterns was also observed, where mCH was accumulated in mature neurons, while adult glial cells had low mCH levels, similarly to the fetal and early postnatal brain (Lister et al 2013). This rapid developmental increase in mCH coincided with synaptogenesis. Moreover, their analysis showed that there is a subset of genes, in both humans and mice, with greater intragenic mCH levels (but not mCG levels) in female neurons compared with males. This mCH signature corresponded to genes that escape the X-inactivation in females (Lister et al 2013).

DNA methylation also plays an important role in cell fate determination during embryonic brain development. For example, DNA methylation was shown to be critical for astrocyte differentiation during mouse brain development (Takizawa et al 2001). Astrocyte differentiation is activated by a transcription factor, STAT3 (Bonni et al 1997). It was demonstrated by Takizawa et al. (2001) that the STAT3 binding region in the glial fibrillary acidic protein (GFAP) promoter is highly methylated on embryonic day 11.5, which abolishes the accessibility of STAT3 and therefore inhibits transcription activity in neuroepithelial cells (Takizawa et al 2001). Later during mouse brain development, on embryonic day 14.5, when

neuroepithelial cells differentiate into astrocytes, the same binding element in GFAP was demethylated in STAT3-responsive cells (Takizawa et al 2001).

DNA methylation in placenta

In contrast to other tissues, the human placenta displays some unique patterns of DNA methylation (Christensen et al 2009, Novakovic & Saffery 2012). The placenta is known to reveal a lower global DNA methylation profile, which may be due to hypomethylation at repetitive elements (Gama-Sosa et al 1983b, Grigoriu et al 2011, Perrin et al 2007). Proper functioning of the DNA methylation machinery is required for normal development of placenta. The most important influence of DNA methylation in the placenta is genomic imprinting (Monk et al 2006).

Changes in DNA methylation in the placenta are associated with low infant birth weight and several pregnancy complications, such as preterm birth, fetal growth restriction, and preeclampsia (Banister et al 2011, Filiberto et al 2011, Kulkarni et al 2011). For example, a recent study showed alterations in the DNA methylation of promoter regions of cortisol-signaling and steroidogenic genes in preeclamptic placentas (Hogg et al 2013). In placentas with early onset preeclampsia, DNA methylation was increased at CpG sites within genes encoding the glucocorticoid receptor and corticotropin releasing hormone (CRH) binding protein, and decreased within CRH in comparison to normal placental tissues (Hogg et al 2013). It is possible that such changes may significantly affect fetal brain development.

Partially methylated domains and their role in brain and placenta

Early studies have revealed that the major portion of the human genome in most tissues is highly methylated (Ehrlich et al 1982). However, it was demonstrated in human fetal and neuronal cell lines that there are large regions within the human genome with partially methylated domains (PMDs) that are associated with inactive chromatin and repressed transcription (Lister et al 2009, Schroeder et al 2011). PMDs cover up to 40% of the genome and are over 100 kb in length in the cell lines. Nevertheless, they are absent in many mature tissues, including cerebral cortex (Maunakea et al 2010, Xin et al 2010). Therefore, it remains an open question whether

PMDs are only a mark of transient cell development stages when cells are not fully differentiated yet, or whether they also exist in mature neuronal tissues (Schroeder et al 2011). Intriguingly, a recent study by Schroeder et al. (2013) showed that PMDs are present not only in cultured cells and cancers, but also in normal tissues, such as full-term human placenta (Schroeder et al 2013). Their results showed that PMDs are stable throughout gestation and between individuals and are enriched in genes with tissue-specific functions. Further functions of PMDs in the human genome are still to be verified (Schroeder et al 2013).

Histone modifications and chromatin remodeling in the brain

Chromatin remodeling and histone modifications play a key role in the accessibility of the nucleosome to the transcription machinery, and thus in gene silencing. The level of gene expression depends on different factors, including modifications in the histone N-terminal tails by methylation, acetylation, phosphorylation, SUMOylation, ubiquitination, and ADP-ribosylation. A pivotal role of chromatin remodeling was established in the inactivation of sex chromosomes as well as in telomere clustering and DNA damage responses (Celeste et al 2003a, Celeste et al 2003b, Fernandez-Capetillo et al 2003a, Fernandez-Capetillo et al 2003b).

Chromatin remodeling and histone modifications are also important factors in brain development and functioning of the nervous system. For example, they were shown to play a role in the conversion of oligodendrocyte precursors to neural stem cells, which can then generate neurons and glial cells (Kondo & Raff 2004). In a mouse model of neurodegenerative conditions in the adult brain, increased histone acetylation by inhibition of histone deacetylases was shown to induce sprouting of dendrites, an increased synapse number, and reinstate cognitive function together with improved retrieval of long-term memories (Fischer et al 2007). Another line of evidence supporting a pivotal role of chromatin remodeling in brain development stems from studies showing that mutations in genes that encode chromatin remodelling factors underlie different forms of X-linked mental retardation (Berube et al 2005, Chelly & Mandel 2001). For example, the chromatin-remodeling protein ATRX was shown to play a critical role in neuronal

survival during brain development in a mouse model (Berube et al 2005). Conditional inactivation of the *Atrx* gene in the embryonic forebrain caused significant neuronal loss during early stages (E11–E13.5) of corticogenesis (Berube et al 2005). For further details on chromatin remodeling in neuronal development and plasticity see (Ho & Crabtree 2010, Hsieh & Gage 2005, Yoo & Crabtree 2009).

Stress and epigenetic regulation

Stress: general terms and definitions

Stress initiates a cascade of biochemical reactions in the body and, depending on its duration and severity, may represent a risk factor for a variety of health complications, including neurological and mental illnesses. The first definition of stress was provided by Walter Bradford Cannon in 1914. According to Cannon, stress "is the body's ability to prepare itself instantaneously to respond to physical threat" (Cannon 1914). Yet the classical definitions of stress originate from the seminal work of Hans Selye, who introduced the concept of the General Adaptation Syndrome in 1936 (Selye 1936). Selye defined stress as "the nonspecific response of the body to any demand made on it" (Selye 1936). According to more contemporary stress researchers like Robert Sapolsky, the negative consequences of stress on health are related to the non-specificity of the stress response (Sapolsky 2000). As noted by Sapolsky, in mammals the stress response evolved to cope mostly with short-term physical stressors (Sapolsky 2000). Therefore, a chronically activated stress response to continuous psychological stressors in day-by-day life may eventually lead to stress-related disease. Effective coping with stress or stress resilience involves not only a rapid activation of physiological and behavioural responses to reinstate the homeostasis, but also an effective termination of the stress response (de Kloet et al 2005).

The stress response can be generally defined as physiological and behavioural adaptation to the emotional or physical threats, or disrupted homeostasis, either actual or anticipated. Stress

may have beneficial, harmless or harmful consequences, depending on many factors, such as duration and intensity of stress, type of stressor, and individual differences in coping with stress. In response to acute stress the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) that activate the pituitary gland. In turn, the pituitary gland releases adrenocorticotrophic hormone (ACTH), which triggers the secretion of the glucocorticoids from the adrenal gland into the bloodstream. Excessive amounts of circulating glucocorticoids (here referred to as CORT – cortisol in primates or corticosterone in most rodents) are beneficial or harmless for a short period of time. However, chronic elevation of CORT due to persistent activation of the hypothalamic-pituitary-adrenal (HPA) axis may lead to the development of various pathological conditions including those that can damage nervous system (Brown et al 2004). The negative effects of chronic stress are not limited to the action of the glucocorticoids. The role of CORT and other hormones and their receptors in the brain in response to stress is discussed below.

Role of stress in brain development

In contrast to the biological effects of man-made chemicals discussed previously, chronic exposure to stress hormones could be considered as a natural source of endocrine disruption. The developing brain is very sensitive to the effects of stress, especially due to the programming properties of glucocorticoids (Seckl 1998). Intriguingly, it was shown *in vitro* using microarray analysis, that chronic CORT exposure of the fetal brain for three weeks alters the expression of 1,648 mRNA transcripts (Salaria et al 2006). This *in vitro* model supports the well accepted concept of the potential contribution of prenatal stress to the reprogramming of the HPA axis and stress response systems in the brain (Harris and Seckl 2011).

Experiments by Henry and colleagues in the mid-nineties used rats to investigate the long-lasting effects of prenatal stress on the functioning of the HPA axis in the offspring at different ages (Henry et al 1995). Pregnant dams were exposed to restraint stress during the third week of gestation and long-lasting outcome on the male offspring at the age of 3, 21 and 90 days

was assessed. The results revealed a significant increase in plasma CORT levels in prenatally stressed offspring at the age of 3 and 21 days after exposure to a novel environment, whereas at the age of 90 days prenatally stressed rats exhibited a longer duration of CORT secretion after novelty exposure when compared to their non-stressed counterparts (Henry et al 1995). Prenatal restraint stress exposure also resulted in the decrease of GR and MR densities in the hippocampus of 21- and 90-day old offspring (Henry et al 1994). In another study the authors also showed that prenatal stress causes long-lasting changes in the dopamine sensitivity of the nucleus accumbens in the adult offspring (Henry et al 1995). Thus, maternal exposure to stress during pregnancy permanently programs the offspring's stress response with potential significant effects on lifelong health trajectories.

The effects of glucocorticoids and other steroid hormones (such as mineralocorticoids, androgens, estrogens, progestrogens, etc.) on the brain cannot be underestimated. The brain is an important target for steroid hormones and any agent that perturbs the delicate hormonal balance can disrupt normal brain development (McEwen 1992, McEwen et al 1979). The importance of steroid hormones in brain development emerges as early as their receptors appear in neurons (McEwen 1987). Most of the brain receptors for steroid hormones belong to the nuclear receptor family and are transcription factors. As transcription factors they recruit many other proteins that are sensitive to epigenetic modifications (McCarthy et al 2009). Thus, as highlighted by McCarthy et al. (2009), steroid hormones and their receptors in the brain represent a unique component within the complex endocrine pathways that is vulnerable to epigenetic regulation during brain development and define adult sex differences in brain and behaviour (McCarthy et al 2009). Besides their action as transcription factors steroid hormone receptors also play an important role in intracellular signaling (Blaustein 2012).

Interestingly, the role of steroid hormone receptors is not limited to their interaction with cognate hormones (Blaustein 2004). Many studies demonstrated that steroid hormone receptors could be activated by neurotransmitters and intracellular signaling systems in the absence of the

respective hormone (Auger 2001, Ciana et al 2003, O'Malley et al 1995, Olesen et al 2005). It has been recognised since the early 1980's that neurotransmitters can regulate the density of steroid hormone receptors in the brain in response to environmental stimuli (McGinnis et al 1985, Nock et al 1981, Thornton et al 1986). Thus, stressful experiences during vulnerable time periods of development, such as the prenatal period, early life or puberty can permanently modulate the brain's response to hormones. For example, experiencing shipment stress during puberty was reported to alter sexual behaviour response to ovarian hormones later in adulthood in mice (Ismail et al 2011, Laroche et al 2009). Extensive studies in human cohorts are outlined in section 6.5.

Notably, long-term consequences of stressful experiences in early postnatal life on hormone receptor expression were shown to be regulated by epigenetic mechanisms (Champagne et al 2006, Weaver et al 2004). Variations in maternal behaviour were shown to be associated with differences in expression of estrogen receptors and glucocorticoid receptors in the brain of the offspring (Champagne et al 2003, Liu et al 1997). Changes in maternal behaviour were shown to be transmitted through generations and were associated with cytosine methylation of promoter regions of hormone receptors (Champagne et al 2006, Francis et al 1999). Epigenetic mechanisms mediating intergenerational and transgenerational programming of maternal behaviour via alteration in the expression of stress hormones are discussed in more detail below.

Experience-dependent programming of stress response and epigenetic marks

A classic example of an environment-epigenome interaction regulating stress responses originated from a series of studies by the laboratories of Michael Meaney and Moshe Szyf using a rat model. Together these studies highlight the importance of early-life experiences in HPA axis development with potentially lasting consequences throughout the lifetime. The authors related the phenotype of maternal care, as exemplified by licking/grooming and arched-back nursing (LG-ABN), during the first 10 days of their offspring's life to their stress response in adulthood. Maternal licking/grooming of pups represents a central aspect of mother-infant interaction. The studies showed that if mothers displayed low LG-ABN behaviour, i.e. a reduced quality of

maternal care, their offspring will show impaired behavioural and physiological coping responses to stress in adulthood as compared to offspring born to mothers with high LG-ABN traits (Caldji et al 2000, Caldji et al 1998). Moreover, the progeny of the low LG-ABN mothers showed decreased open-field exploration as well as longer latencies to begin eating food in a novel environment, paired with increased levels of corticotropin-releasing hormone (CRH) receptors in the amygdala (Caldji et al 1998). On the other hand, the offspring of high LG-ABN showed reduced responses to restraint stress, such as reduced plasma adrenocorticotropic hormone and corticosterone, in adulthood along with increased hippocampal glucocorticoid receptor (GR) density and decreased hypothalamic CRH mRNA level (Liu et al 1997).

Interestingly, these studies demonstrated evidence of epigenetic transmission of the maternal phenotype of LG-ABN behaviours in a set of cross-fostering experiments (Francis et al 1999). The alteration in the stress response system observed in the progeny of low LG-ABN can be reversed if those animals were raised by high LG-ABN and *vice versa* (Francis et al 1999). The reversal of impaired stress response programming by rat pups born to a low LG-ABN mothers and fostered by a high LG-ABN mother indicates that maternal care alters the stress response independently of information passed on in the womb, but rather through postnatal environmental conditions. An important finding in this context was that increased levels of GR mRNA in the hippocampus of the offspring of the high LG-ABN were due to low levels of DNA methylation at the exon 1_7 of the GR promoter region (Weaver et al 2004). This was in contrast to the observed DNA methylation pattern in the hippocampus of the offspring of low LG-ABN, where exon 1_7 of the GR promoter region was always methylated (Weaver et al 2004). Intriguingly, the authors provided evidence of non-germ line transmission of the maternal behaviour to the adopted offspring via epigenetic mechanisms. Cross-fostering experiments showed that the DNA methylation status of the exon 1_7 promoter of the GR gene depends on the rearing condition rather than the genetic background (Weaver et al 2004). In the offspring of low LG-ABN mothers raised by high LG-ABN mothers the DNA methylation status within 5' CpG dinucleotide of the 1_7 GR

promoter region was indistinguishable from that of the biological offspring of high LG-ABN (Weaver et al 2004). Similar evidence of non-genomic transmission was observed when biological offspring of high LG-ABN was fostered to the low LG-ABN mothers (Weaver et al 2004). Notably, the distinctive maternal care traits can be transmitted to the subsequent generations of female offspring (Francis et al 1999) potentially through epigenetic mechanisms (Ward et al 2013; Zucchi et al 2013).

This series of studies of maternal care also demonstrated altered histone acetylation and binding of the transcription factor (NGFI-A) to the GR promoter. The adult offspring of high LG-ABN mothers displayed an increase in histone H3-K9 acetylation as well as a three-fold increase in the binding of NGFI-A protein to exon 1_7 of the GR promoter in the hippocampus (Weaver et al 2004). Interestingly, central infusion of histone deacetylase inhibitor, trichostatin A (TSA), reversed the observed epigenetic changes and eliminated the maternal effect on HPA responses to stress in the offspring of the low LG-ABN mothers (Weaver et al 2004). Intracerebroventricular infusion of TSA into the adult offspring of LG-ABN mothers abolished the hypermethylated state of the GR promoter and removed the differences in GR expression, histone acetylation and NGFI-A binding in the hippocampus between the progenies of low- and high LG-ABN mothers (Weaver et al 2004). Thus, these studies highlight a crucial role of early-life experience in life-time stress responses via epigenetic changes. However, more experiments are required to verify the exact molecular mechanisms of how maternal behaviour is able to produce stable alterations in the DNA methylation and chromatin structure in the offspring (Weaver et al 2004, Weaver et al 2007). One of the proposed mechanisms of regulation of GR transcription in the hippocampus involves a thyroid hormone-serotonin-NGFI-A signalling cascade (Hellstrom et al 2012). Hellstrom et al. (2012) demonstrated that thyroid hormones and serotonin (5-HT) are key mediators of the effects of both, pup licking and grooming and tactile stimulation, on NGFI-A binding to the exon 1_7 of the hippocampal GR promoter.

More translational research is required to evaluate the causal mechanisms that lead to benefits of tactile stimulation. Interestingly, a recent human study by Sharp et al (2012) investigated whether self-reported maternal stroking over the first weeks of life has beneficial effects on the offspring, similar to those observed as a result of high licking/grooming in rodents (Sharp et al 2012). This study found an association between increased maternal depression and decreased physiological adaptability, accompanied with increased negative emotionality in the offspring, only in the presence of low frequency of maternal stroking (Sharp et al 2012). In another translational study, the authors investigated whether maternal depression/anxiety during third trimester in humans has an effect on methylation of NR3C1 at a predicted NGFI-A binding site in the infant (Oberlander et al 2008). Similarly to findings in animal models, Oberlander et al. (2008) found that prenatal exposure to increased maternal depression/anxiety was associated with an increased methylation at a predicted NGFI-A binding site, paired with an increase in salivary cortisol stress responses at the age of three months in the infant.

Another example of how early life experience can induce long-lasting changes in physiology and behavior comes from a mouse model (Murgatroyd et al 2009). Murgatroyd et al. (2009) demonstrated that exposure to stress during first 10 days of life caused corticosterone hypersecretion under basal conditions and in response to acute stressors, at the age of six weeks, three months and one year in mice. In addition, they observed increased arginine vasopressin (AVP) expression in the hypothalamic paraventricular nucleus that was associated with DNA hypomethylation of the enhancer of *Avp* gene (Murgatroyd et al 2009). Their results indicated that the Avp enhancer contains high-affinity DNA-binding sites for CpG binding protein 2 (MeCP2) that regulates activity-dependent transcription of the *Avp* gene, and phosphorylation of MeCP2 prevents Avp enhancer occupancy (Murgatroyd et al 2009). Furthermore, molecular changes in the brain of mice exposed to early-life stress were accompanied by an altered behavioural phenotype. The stressed mice demonstrated increased immobility in the forced swim test and memory deficits in an inhibitory avoidance task (Murgatroyd et al 2009). Taken together, the

above mentioned studies highlight a pivotal role of early-life experiences in the alteration of stress response systems later in life that are in part regulated by epigenetic mechanisms.

Stress-induced epigenetic changes in placenta may be associated with epigenetic changes in brain

According to the examples outlined in the previous chapters, stress or other types of adverse experience will modify epigenetic profiles in the placenta and brain. In a recent report by Howerton et al. (2013), O-linked-N-acetylglucosamine transferase (OGT) has been identified as a placental biomarker of maternal stress (Howerton et al 2013). OGT is an enzyme that catalyzes the addition of a single N-acetylglucosamine in O-glycosidic linkage to serine or threonine residues of intracellular proteins. OGT is an X-linked gene that plays an important role in regulating proteins involved in chromatin remodeling. In mice, levels of both OGT and its biochemical mark O-GlcNAcylation in placental tissue showed a sex-specific pattern with low expression in males and a further reduction by prenatal stress (Howerton et al 2013). Importantly, OGT levels in human placenta showed a similar sexually dimorphic pattern (Howerton et al 2013).

A central question in the search for new biomarkers of disease is if epigenetic changes in the placenta reflect functional and/or epigenetic changes in the developing organs such as the brain. A recent study by Jensen Pena et al. (2012) assessed the role of epigenetic mechanisms in the placenta and fetal brain in response to prenatal stress in rats (Jensen Pena et al 2012). The study focused on the expression of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), which catalyzes the interconversion of active glucocorticoids (such as corticosterone in rats) and inert 11-keto forms (cortisone, 11-dehydrocorticosterone) in placenta and fetal brain in response to chronic stress. Chronic restraint stress during gestational days 14-20 caused a significant decrease in *Hsd11b2* mRNA, paired with an increase in DNA methylation at CpG sites within the *Hsd11b2* gene promoter in placenta (Jensen Pena et al 2012). The authors also found an increased level of *Dnmt3a* mRNA in the fetal hypothalamus, as well as decreased methylation at *Hsd11b2*.

However, the level of *Hsd11b2* mRNA in the embryonic hypothalamus was not altered (Jensen Pena et al 2012).

It is worth noting that fetuses and placenta in the latter study were obtained via caesarian section. It was demonstrated recently that caesarian section can alter the epigenetic state of neonatal hematopoietic stem cells (Almgren et al 2014). Infants delivered by caserian section presented with increased global DNA methylation in CD34+ cells in comparison to those that were delivered vaginally. In addition, locus-specific analysis showed that 76% of the differentially methylated loci in neonatal CD34+ cells were hypermethylated after vaginal delivery (Almgren et al 2014). Thus, this recent study highlights the possibility that caesarian section by itself may represent an additional contributing factor that determines the epigenetic states of the offspring in addition to prenatal stress and other factors.

The involvement of epigenetic mechanisms in the placental and brain stress response has been previously demonstrated in mice (Mueller & Bale 2008). In this study, prenatal stress caused significant sex-specific elevation of DNMT1 expression in female placentas. Interestingly, males but not females displayed maladaptive behavioural stress responsiveness paired with altered methylation and gene expression of glucocorticoid receptor and corticotropin-releasing factor in the fetal brain (Mueller & Bale 2008). Furthermore, prenatal stress resulted in a sex-specific increase in the placental expression of PPARα (peroxisome proliferator-activated receptor α), IGFBP-1 (insulin-like growth factor binding protein 1), HIF3α (hypoxia-inducible factor 3α) and GLUT4 (glucose transporter 4) in males (Mueller & Bale 2008). These studies suggest that placental epigenetic patterns may serve as predictive signatures of stress response in later life, however, the mechanisms of this programming and their consequences on life health trajectories still remain to be investigated.

Stress-induced epigenetic signatures of disease

miRNAs as markers of stress and disease

The role of miRNAs in stress responses in general has been extensively studied (reviewed in (Leung & Sharp 2010)), including their role in the brain under stressful conditions in particular (Schouten et al 2013). Research from our laboratory showed that even very mild psychological stress can induce long-lasting changes in miRNA expression in the rat brain (Babenko et al 2012a). We demonstrated that expression of miR-186 and miR-709 in the brain was altered in response to restraint stress. In our experiment adult male rats were stressed for 20 minutes daily for two weeks (Babenko et al 2012a). MiRNA expression analysis showed that miR-186 and miR-709 remained altered in the prefrontal cortex even when animals had two weeks of recovery from chronic stress (Babenko et al 2012a). Interestingly, in a separate study we found that expression of miR-186 was also altered in the frontal cortex of rat mothers that experienced stress during late gestation (Zucchi et al 2013). Furthermore, the latter study found that prenatal stress in the offspring induces miRNA signatures linked to neurological and psychiatric disorders in humans, which indicates that early adverse experiences are associated with potentially long-term epigenetic biomarkers in the brain (Zucchi et al 2012, Zucchi et al 2013). It seems reasonable to expect that some of these miRNA signatures of stress may transmit to subsequent generations of progeny with potentially adaptive or maladaptive consequences on behaviour and endocrinology. The critical role of miRNAs in programming lifetime health in response to an adverse prenatal environment and their impact across generations has not yet been systematically explored and still remains underappreciated.

MiRNAs were implicated in many diseases of the nervous system, including stroke, Alzheimer's disease, Parkinson's diseases, Huntington's disease, multiple sclerosis, schizophrenia, autism, anxiety, depression and bipolar disorder (reviewed by (Babenko et al 2012b), (Miller & Wahlestedt 2010)). The most intriguing conclusion of previous studies is the emerging role of circulating miRNAs as potential biomarkers for diagnostics and prognosis of

CNS diseases (Jin et al 2013). Despite the fact that epigenetic inheritance is receiving increasing attention during the recent decades, the transgenerational inheritance of miRNAs was not yet demonstrated in mammals.

Changes in brain DNA methylation in response to stress and disease

Many studies in both humans and animal models pointed out that stress has multiple effects on DNA methylation. For example, a study by Unternaehrer et al. (2012) examined whether acute psychological stress in humans causes changes in DNA methylation of genes related to brain plasticity and endocrine regulation, such as brain-derived neurotrophic factor (*Bdnf*) and oxytocin receptor (*Oxtr*) (Unternaehrer et al 2012). The study includes the results for 76 participants of 61-67 years old that underwent the Trier social stress test. Comparisons of quantitative DNA methylation pattern in whole blood before stress, 10- and 90 min after the experience of stress revealed increased methylation of the *Oxtr* gene pre-stress compared to 10 min post-stress and decreased methylation from 10 min post-stress compared to 90 min post-stress. These observations reflect the dynamic nature of DNA methylation regulation in response to psychological stress. By contrast, no changes in DNA methylation were observed at the examined *Bdnf* region (Unternaehrer et al 2012). Interestingly, Roth et al. (2009) used a rodent model of childhood maltreatment to investigate how adverse early-life experience influences DNA methylation of the *Bdnf* gene. For the first week of postnatal life new-born rat pups were exposed to stressed caretakers that predominately displayed abusive behaviors. Their results showed long-lasting increase in the *Bdnf* DNA methylation pattern in the adult prefrontal cortex (Roth et al 2009). Similar changes were observed in the offspring of female rats exposed to adversity in infancy (Roth et al 2009). In addition, animals exposed to abusive behaviour in early childhood had significantly less *Bdnf* mRNA expression in prefrontal cortex throughout the life span, which was accompanied by an increase in DNA methylation at various regions of *Bdnf* gene (Roth et al 2009). It is worth noting that maltreatment-induced *Bdnf* expression deficiency was reversed by chronic infusion of zebularine, a DNA methylation inhibitor (Roth et al 2009).

In line with these studies, Sterrenburg et al. (2011) used an animal model of chronic stress to investigate DNA methylation changes in another stress-related gene, encoding corticotropin-releasing factor (CRF). Their results showed that chronic variable mild stress causes site-specific DNA methylation changes in various parts of the brain (Sterrenburg et al 2011).

In humans, a recent study by Fuchikami et al. (2011) investigated methylation profiles of the *Bdnf* gene in patients with major depression. Analysis of methylation profiles of CpG units within *Bdnf* promoter using genomic DNA from peripheral blood allowed distinguishing between patients with major depression and healthy controls in concordance with clinical diagnoses (Fuchikami et al 2011). Hence these findings indicate that DNA methylation profiles of the BDNF gene may serve as a diagnostic biomarker of major depression. Changes in the DNA methylation profiles of peripheral blood were also demonstrated for a number of other genes in patients suffering from major depression (Rotter et al 2011, Uddin et al 2011).

Aberrant DNA methylation is also linked to other mental illnesses, such as schizophrenia, bipolar disorder, major psychosis, autism spectrum disorders (Dempster et al 2011, Kuratomi et al 2008, Mill et al 2008, Nguyen et al 2010). For example, analysis of saliva of patients with schizophrenia and bipolar disorder showed hypomethylation of the serotonin receptor type-2A gene (Ghadirivasfi et al 2011). Hypomethylation of another gene, reelin, was found in the post-mortem cortices of schizophrenia patients (Grayson et al 2005). Using whole blood samples from schizophrenia patients, Liu et al (2013) reported that DNA methylation changes in the blood may play a protective role and reduce delusion and hallucination symptoms in patients (Liu et al 2013). The authors found 11 CpG sites with altered methylation patterns in the patients that significantly correlated with reality distortion symptoms (Liu et al 2013). Taking into consideration that this study may be confounded by various proportions of leukocyte subtypes in the whole blood samples, further investigations are needed. It is worth noting that DNA methylation of brain tissues may be more directly related to schizophrenic symptoms than DNA methylation from

peripheral blood of the patients (Liu et al 2013). These findings emphasize the potential predictive or diagnostic value of epigenetic markers in human mental and neurological disease.

Stress during pregnancy and risks of neurological disorders later in life

As discussed above, stress can play an important regulatory role in both brain development and disease etiology. On the other hand, several lines of evidence point towards the role of stressful experiences in transgenerational programming and their critical ability to alter epigenetic regulation. Thus, we hypothesise that stress during pregnancy may affect mental health in the offspring across a lifetime and across multiple generations through heritable changes at the epigenetic level. Below we review the role of stress in relation to the risk of neurological disorders later in life and discuss evidence that suggests a causal role for epigenetic mechanisms in the predisposition to mental illness.

Many previous clinical and animal studies suggested a causal association between an adverse prenatal environment and an elevated risk of cardiovascular disorders and psychiatric diseases later in life (Cottrell & Seckl 2009). Interestingly, a recent study by Booij et al. (2012) investigated whether perinatal adversity in humans has long-term consequences on central serotonin neurotransmission in adulthood (Booij et al 2012). Their results showed that a history of obstetric complications, such as a delivery with signs of fetal physiological distress, predicted lower brain serotonin synthesis in the medial orbitofrontal cortex and hippocampus in 27-year-old adults (Booij et al 2012). Since serotonin neurotransmission in these brain regions is involved in emotional regulation, affective state and stress coping, these findings support the notion that a reduction in serotonin activity caused by perinatal stressors may contribute to the susceptibility for psychiatric disorders in later life (Booij et al 2010).

One approach to investigate the role of prenatal stress on the offspring's future health is to study the effect of prenatal exposure to exogenous steroids in obstetrics [reviewed in (Schwab 2009, Sloboda et al 2005)]. Synthetic steroids, such as dexamethasone or betamethasone, are administered to about 6% of pregnant women who are at risk of premature labour to facilitate

fetal lung maturation (Mahony et al 2010). Notably, about half of the treated pregnant women do not deliver within 7 days (Boesveld et al 2014). While in children at 5 years of age conclusive evidence of effects of antenatal corticosteroids on neurodevelopmental outcomes could not be confirmed (Asztalos et al 2013), long-lasting effects of antenatal corticosteroid treatment in term-born children revealed increased cortisol reactivity in response to acute stress at the age of 6 to 11 years (Alexander et al 2012). In sheep such developmental consequences of antenatal dexamethasone treatment were linked to reduced fetal cerebral blood flow and altered fetal electrocortical activity (Schwab et al 2000, Schwab et al 2001).

Another approach investigated the short- versus long-term consequences of stress experienced by the mother during pregnancy on the future health of the offspring. Epidemiological studies revealed some links between stress experienced by the mother during pregnancy and higher risk of developing depression, anxiety, schizophrenia, ADHD and autism in the offspring (Kinney et al 2008b, Markham & Koenig 2011). Some human data and animal studies also suggest a critical role of prenatal stress in developing anxiety- and depression-related behaviours later in life (Markham & Koenig 2011). Further details linking perinatal stress to future mental health and wellbeing in the offspring are discussed in the following chapter.

Prenatal stress and risk of developing schizophrenia later in life

Schizophrenia is likely multifactorial in origin, however, an adverse maternal environment during fetal development arguably represents a predisposing or precipitating factor for this mental disorder. Several epidemiological studies reported a relationship between maternal psychological stress and an elevated risk of schizophrenia in the adult offspring (Huttunen & Niskanen 1978, Khashan et al 2008, Khashan et al 2011b, Kinney et al 1999b, Myhrman et al 1996b, Selten et al 1999a, van Os & Selten 1998b) (Figure 1.2). Growing evidence supports the hypothesis that at least one form of schizophrenia has a neurodevelopmental cause and has its origins in prenatal disturbances (Watson et al 1999).

Evidence of this relationship derives from traumatic war experiences that occurred during pregnancy. A study by van Os et al. (1998) investigated the lifetime risk for developing schizophrenia in individuals whose mothers were pregnant during the German invasion in the Netherlands in the 1940s. The incidence of developing schizophrenia was higher in the offspring of mothers who experienced the invasion during pregnancy in comparison to those unexposed (van Os & Selten 1998b). Furthermore, Malaspina (2008) reported a raised incidence of schizophrenia in offspring whose mothers were pregnant during the Arab-Israeli war of 1967 (Malaspina et al 2008). By contrast, more recent study by Selten at al. (2003) failed to find an association between prenatal exposure to stress during major wars in Israel and subsequent risk of schizophrenia (Selten et al 2003).

The risk to develop schizophrenia may be influenced by the timing of adverse experiences in relation to critical periods of brain development. A retrospective epidemiological study by Huttunen and Niskanen (1978) suggested for the first time that children, whose fathers had died before their birth had higher risks of developing schizophrenia than those children whose fathers died during the first year of their lives. Subjects who were still *in utero* during the time when they lost their fathers were six times more likely to be hospitalized due to their schizophrenia than respective controls (Huttunen & Niskanen 1978). These findings indicate that prenatal stress may have more potent effects on brain development than postnatal experiences. A more recent population-based studies by Khashan et al. (2008, 2011) showed that the risk of schizophrenia was elevated in offspring whose mothers were exposed to the death of a relative during the first trimester (Khashan et al 2008, Khashan et al 2011b). These studies indicate that prenatal stress may elevate the risk of psychiatric disorders in the offspring as a function of pregnancy stage (Buss et al., 2012; Zhu et al., 2013).

In addition to this intriguing human evidence, animal studies also highlighted the role of prenatal stress in a schizophrenia-like phenotype (Fig. 1.2). For example, Markham et al. (2013) demonstrated that prenatal stress exposure during late gestation causes sex-specific alterations in

the maturation of the prefrontal cortex during adolescence in rats. Male but not female rats had signs of disrupted maturation of the apical dendrites in the prefrontal cortex (Markham et al 2013).

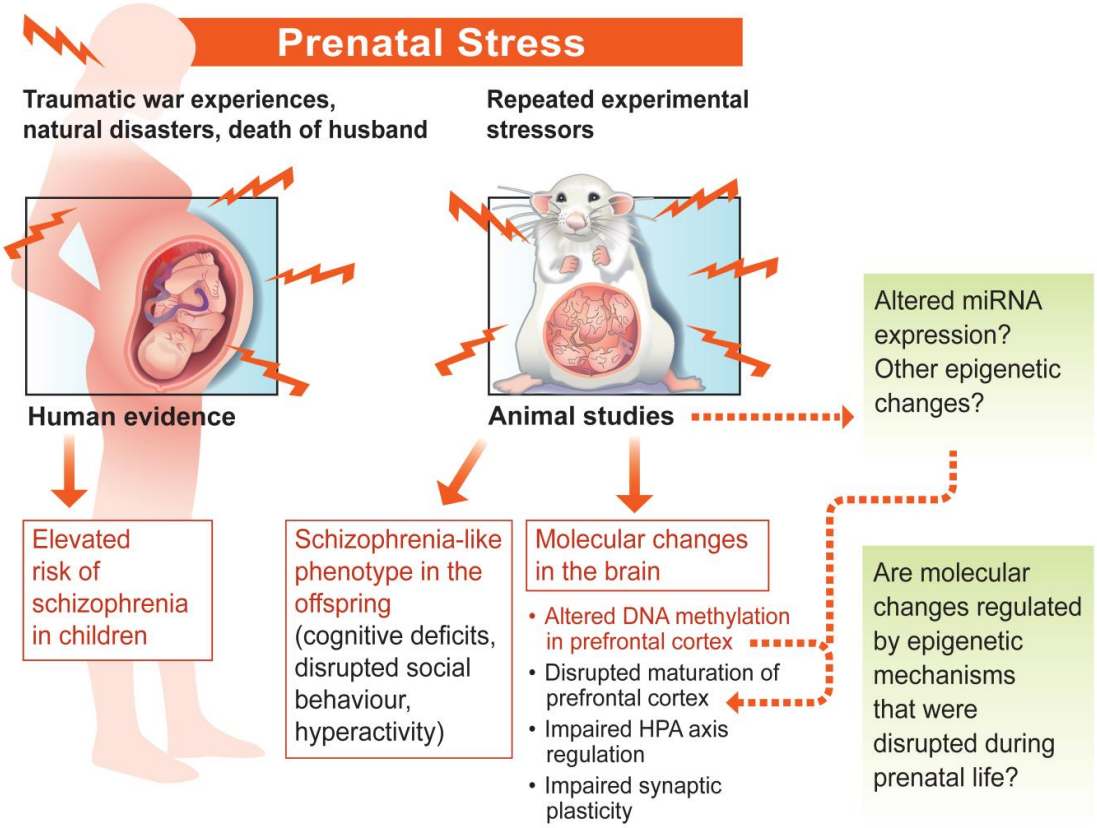


Figure 1.2. Prenatal stress and risk of developing schizophrenia later in life (graphic support, Amanda McRoberts). Evidence from human observations indicated that stressful experiences during pregnancy are associated with higher risk of schizophrenia in children. On the other hand, studies in rodents demonstrated that a schizophrenia-like phenotype in prenatally stressed offspring is paired with molecular changes in the brain, including altered DNA methylation in the prefrontal cortex. The involvement of other epigenetic mechanisms, such as altered miRNA expression, in a schizophrenia-like phenotype remains to be characterized.

These findings are in line with reports that suggested a causal role for prenatal stress in a schizophrenia-like phenotype and cognitive deficits, including hypersensitivity to amphetamine, blunted sensory gating, disrupted social behavior, impaired HPA axis regulation, and aberrant expression of genes involved in synaptic plasticity in the prefrontal cortex (Kinnunen et al 2003, Koenig et al 2005, Lee et al 2007, Markham et al 2010). Recently, prenatal stress has also been

shown to cause schizophrenia-like behavioural changes and molecular alterations in expression of serotonin 2A and metabotropic glutamate 2 receptors in the mouse adult frontal cortex (Holloway et al 2013).

Interestingly, prenatal stress in mice was shown to induce alterations in DNA methylation paired with a schizophrenia-like phenotype (Matrisciano et al 2013). Offspring born to non-stressed mothers had high levels of *DNMT1* and *DNMT3a* mRNA expression in the frontal cortex at birth, but these levels progressively decreased at postnatal days 7, 14, and 60 (Matrisciano et al 2013). This is in contrast to prenatally stressed offspring that displayed high levels of DNMTs compared to controls at all time-points (Matrisciano et al 2013). These prenatally stressed mice showed hyperactivity and deficits in social interaction, prepulse inhibition, and fear conditioning in adulthood. Interestingly, the deficits were corrected by administration of a histone deacetylase inhibitor, valproic acid, and by clozapine, which acts as an antipsychotic agent with DNA-demethylation activity (Matrisciano et al 2013). These findings propose a causal function of DNA methylation underlying the cognitive deficits of a schizophrenia-like phenotype.

Prenatal stress and risk of anxiety- and depression-related disorders later in life

Exposure to prenatal stress represents a major determinant of lifelong affective and emotional wellbeing. In humans, several epidemiological studies pointed out a link between prenatal stress and risk of anxiety- and depression-related disorders later in life (Brown et al 2000, O'Connor et al 2002b, O'Connor et al 2003, Torrey et al 1996, van den Bergh et al 2008, Watson et al 1999). For example, Watson et al. (1999) reported that 18-year-old males but not females, exposed *in utero* during the second trimester of gestation to the Tangshan earthquake in China in 1976, present with a significantly increased risk of severe depression (Watson et al 1999). Exposed subjects had significantly more depressive symptoms when compared to a control group of non-exposed students (Watson et al 1999).

Aside from prenatal stress *per se*, maternal mental illness also bears the risk to affect mental health in her children, possibly through routes of behavioural and/or endocrine

programming. For example, O'Connor et al. (2003) investigated the influence of maternal anxiety and depression on behavioural abnormalities in 6-7 year-old children. Their results showed that offspring born to mothers who experienced high levels of anxiety during pregnancy had higher rates of behavioural/emotional problems, as reported by the parents (O'Connor et al 2003). Further, Van den Bergh et al. (2008) found a striking link between maternal anxiety during pregnancy and self-reported depressive symptoms in 14- and 15-year-old adolescents (van den Bergh et al 2008). The authors used the State Trait Anxiety Inventory to establish the levels of maternal anxiety during pregnancy. Their results indicated that maternal anxiety during the 12-22nd weeks of pregnancy was associated with altered cortisol profiles in the offspring. Furthermore, in girls, but not boys, the flattened cortisol profile was also associated with depressive symptoms, as measured using the Children's Depression Inventory (van den Bergh et al 2008). An interesting set of studies by Yehuda and colleagues investigated the consequences of prenatal stress exposure during the World Trade Center attacks on the future health of both mothers and their babies (Yehuda 2002, Yehuda et al 2009, Yehuda et al 2005). Their data suggest that stress during pregnancy is associated with subsequent formation of posttraumatic stress disorder (PTSD) in mothers. In turn, the mother's PTSD was linked to lower cortisol levels in their babies very early in life, thus highlighting the importance of *in utero* contributors to putative biological risk for PTSD (Yehuda et al 2005).

In addition to human data, growing evidence from animal studies supports the notion that depression-like and anxiety-like behaviours in adulthood may have neurodevelopmental origins. For example, the role of prenatal stress in causing depression-like behaviour in rats has been recognized since the early nineties (Alonso et al 1991). The study by Alonso and colleagues (1991) demonstrated that adult female rats, when exposed to stress while *in utero*, display more depressive-like behaviour in a forced swimming task (Alonso et al 1991). Secoli and Teixeira also reported that chronic prenatal stress caused behavioural symptoms of depression in a rat model

(Secoli & Teixeira 1998). These studies indicate a mechanistic link between prenatal stress exposure and an elevated risk of altered affective state.

Anxiety and other behavioural changes linked to prenatal stress were shown by Vallee et al. (1997), who compared the effects of prenatal stress versus postnatal handling on rat behaviour in adulthood (Vallee et al 1997a). In this study, *in utero* stressed rats demonstrated high anxiety-like behaviour, which correlated with elevated secretion of corticosterone in response to stress. On the other hand, postnatally handled rats showed low anxiety-like behavior, which correlated with low levels of circulating plasma corticosterone levels in response to stress in adulthood. Interestingly, neither prenatal nor postnatal stresses altered spatial learning or memory performance in these animals (Vallee et al 1997a). In a more recent study by Kapoor and Matthews (2005), the authors examined the long-term consequences of prenatal stress on adult male offspring in a guinea pig model (Kapoor & Matthews 2005). Pregnant guinea pigs were exposed to a strobe light for 2 h on different days during late gestation. The results showed that prenatal stress caused a long-lasting decrease in the body weight, as well as elevated plasma cortisol levels in a adrenocorticotropic hormone challenge, and signs of anxiety in prenatally stressed offspring when compared to non-stressed controls (Kapoor & Matthews 2005).

Using a selective breeding line, Bosch et al. (2006) investigated whether prenatal stress can modulate genetic predisposition to anxiety-like behaviour in rats (Bosch et al 2006). Their results showed that rats bred for high (HAB) or low (LAB) anxiety-related behaviour were differentially affected by prenatal stress during gestational days 4-18. Interestingly, prenatally stressed HAB rats became less anxious in adulthood, whereas LAB rats became more anxious, when compared to their respective non-stressed controls. Prenatally stressed HAB rats had decreased levels of CRH mRNA in the paraventricular nucleus of the hypothalamus, whereas prenatally stressed LAB rats showed an increase in hypothalamic vasopressin mRNA expression, when compared to their respective controls (Bosch et al 2006).

Various studies have investigated the mechanisms of such behavioural alterations. For example, depressive-like behaviour, as indicated by increased immobility time in the forced swimming task, exhibited by prenatally stressed adult rats, might be mediated in part through the down-regulation of GR (Karandrea et al 2002) and MR (Tamura et al 2011) in the hippocampus. In the latter study, pregnant dams were exposed to daily restraint stress during late gestation (Tamura et al 2011). The results revealed that the offspring of stressed mothers had reduced dendritic complexity and spine density in neonatal granule cells, which persisted into adulthood (Tamura et al 2011). Interestingly, it was shown previously that a single 20-min restraint stress exposure of a pregnant rat on day 18 after mating leads to a sex-specific reduction of hippocampal granule cells in adult female offspring (Schmitz et al 2002). Thus, even short-term prenatal stress may already lead to sexually dimorphic effects on neuronal morphology in areas relevant to stress response regulation and cognitive performance.

Further animal studies highlighted the pivotal role of the placenta in prenatal reprogramming of the stress response (Welberg et al 2000, Welberg et al 2005). To demonstrate placental contribution to the adult alterations of the HPA axis and anxiety-like behaviour, Welberg et al. (2000) inhibited feto-placental 11beta-hydroxysteroid dehydrogenase type 2 (HSD11B2) in rats, using carbenoxolone (CBX) (Welberg et al 2000). The enzyme HSD11B2 assumes a critical role in the placenta that breaks down maternal cortisol or corticosterone and thus prevents them from crossing the placenta. Thus, HSD11B2 serves a major protective role for the developing fetal brain by rapidly inactivating the main stress hormones (Yang 1997). In the studies by Welberg and colleagues (2000, 2005), CBX treatment of pregnant dams reduced fetal birth weight as well as offspring weight in adulthood. Moreover, adult offspring of CBX-treated rats displayed increased basal corticosterone levels and CRH, as well as reduced GR mRNA in the paraventricular nucleus of the hypothalamus and increased GR mRNA expression in the amygdala (Welberg et al 2000). Interestingly, no changes in either GR or MR were observed in the hippocampus of adult offspring of CBX-treated dams when compared to the offspring of

untreated controls. The results of the open field and forced swim tests suggest that disturbance of the feto-placental barrier to maternal corticosterone produced anxiety-like behaviours in adult offspring in stressful situations (Welberg et al 2000).

In a follow-up study, Welberg et al. (2005) compared the effects of acute versus chronic restraint stress during the third week of gestation on placental HSD11B2 activity in rats (Welberg et al 2005). Their results showed that acute stress up-regulates HSD11B2 activity by 160% in placenta, thus protecting the fetus from excessive levels of maternal corticosterone. Interestingly, chronic stress exposure did not have a significant effect on placental HSD11B2 activity when compared with unstressed pregnant rats, however, it reduced the ability to increase the placental HSD11B2 activity in the face of an acute stressor (Welberg et al 2005). These studies support the general notion that the effects of prenatal stress depend on its duration and severity.

Interestingly, a study by Uddin et al. (2010) suggests that mechanisms of epigenetic regulation are altered in the patients with lifetime depression (Uddin et al 2011). Their results showed that genome-wide methylation profiles are different in depressed versus non-depressed individuals in a community-based setting (Uddin et al 2011). Another epidemiological study by Fuchikami et al. (2011) suggests that DNA methylation profiles of CpG (island I) of the *Bdnf* gene may serve as a diagnostic biomarker for major depression (Fuchikami et al 2011). Thus, these studies indicate a close interplay between epigenetic regulation and depression. For a more detailed review see (El-Sayed et al 2012).

Prenatal stress and predisposition to attention deficit hyperactivity disorder and autism

Attention deficit hyperactivity disorder (ADHD) and autism are two conditions that are significantly influenced by adverse environmental conditions, such as stress. For instance, Rodriguez and Bohlim (2005) investigated the association between maternal smoking during pregnancy and perceived stress with the risks of ADHD in 7-year-old offspring (Rodriguez & Bohlin 2005). Results of multiple regression analysis showed that prenatal stress and exposure to maternal smoking were independently associated with the symptoms of ADHD in the offspring

later in life. The results of logistic regression analysis revealed that stress during pregnancy contributed to ADHD diagnostic criteria, especially in the boys. In particular, the levels of perceived stress during pregnancy predicted nearly 87% of the ADHD cases in the male population studied (Rodriguez & Bohlin 2005). Ronald et al. (2010) reported that maternal stressful events, such as a divorce or a residential move, during pregnancy significantly predicted ADHD behaviors and autistic traits in the 2-year-old offspring, both males and females (Ronald et al 2010). Similarly, Grizenko et al. (2012) showed that mothers with an ADHD-affected child have been more likely to perceive high stress during pregnancy when compared to an unaffected sibling. Moreover, the authors found that the DRD4 7/7 genotype was associated with more severe symptoms of ADHD in the offspring of the mothers who were highly stressed during pregnancy (Grizenko et al 2012).

Another study investigated the link between maternal state anxiety during pregnancy and later ADHD deficits in the 15-year-old offspring (Van Den Bergh et al 2006). State anxiety is a measure of the intensity of transitory anxiety in response to real-life stress and is characterized by perceived tension as well as an increased activity of the autonomous nervous system (Van Den Bergh et al 2006). The results showed that adolescent boys, but not girls, whose mothers experienced high levels of anxiety during pregnancy, had more difficulties with the sustained attention/self-regulation than boys whose mothers reported low or moderate anxiety levels in the State Trait Anxiety Inventory (Van Den Bergh et al 2006).

Many insights into the developmental origins of health and disease are derived from the study of natural disasters occurring during pregnancy (King et al 2012). For example, using the Louisiana state cohort Kinney et al. (2008a) found a significantly higher prevalence of autism disorder (AD) in children whose mothers experienced hurricanes or severe tropical storms during pregnancy (Kinney et al 2008a). Interestingly, the negative influence of natural disasters on offspring health was dose-dependent. The severity of the disaster was assessed using two storm factors: the intensity of a storm's impact on a parish, and the vulnerability of the residents of a

parish to a storm's effects (Kinney et al 2008). AD prevalence (of 6.06 per 10,000 births) was higher in children exposed *in utero* to one or the other storm factor in comparison to the control cohort that had no storm exposure where the prevalence was 4.49 AD cases per 10,000 births. Notably, the prevalence of AD in children exposed *in utero* to both storm factors was 13.32 (Kinney et al 2008a). It is worth noting that children who were exposed to the storm during 5-6 months or the last month of gestation had 3.83 times higher risk of developing AD than those with a different timing of exposure (Kinney et al 2008a).

Possible involvement of the epigenetic alterations in the etiology of AD and ADHD has been suggested (Mill & Petronis 2008, Schanen 2006), however the exact mechanisms are yet to be identified.

Conclusion

The studies discussed in this review highlight the susceptibility of the fetal brain to an adverse maternal environment during a particularly vulnerable period in life through mechanisms that are associated and potentially even mediated by epigenetic regulation. The extent to which maternal stress and anxiety during pregnancy contribute to the development of mental and psychiatric conditions in the child is still far from being understood. However, effective stress management strategies that allow reducing, preventing and effective coping with stress and anxiety may be of great importance for the health of both pregnant women and offspring. The consideration of prenatal stress effects in disease outcomes is critically important to realistically improve current prevention and intervention strategies and assist a healthy life trajectory. Such evidence-based decision making is critical for developing recommendations for a life style that supports healthy development and successful aging in the presence of a stressful environment. Because altered epigenetic regulation may potentially be reversible, the identification of epigenetic signatures of disease presents a promising diagnostic and therapeutic avenue for generations to come.

Objectives and hypothesis

The main goal of the current thesis was to investigate the role of epigenetic changes in the transgenerational stress responses in rats.

Hypothesis: Based on existing evidence and our previous studies, **we hypothesised** that prenatal stress causes transgenerational changes in gene and miRNA expression, as well as in DNA methylation patterns in the developing brain. **We predict** that changes in the brain are associated with and reflected by epigenetic changes in placenta.

To achieve the main goal and asses the transgenerational effects of stress we used four generations of Long-Evans rats. The parental (F0) generation of dams was stressed during pregnancy and then three subsequent generations of offspring were analyzed. In addition, our aim was to test possible cumulative effects of prenatal stress on the offspring. To accomplish this goal we stressed the pregnant dams in each generation (F0, F1, and F2) and assessed the effects of prenatal stress on their offspring in F1, F2, and F3 generations.

Three experiments were conducted to achieve the main goal and test the overall hypothesis, as outlined below.

Experiment 1 (Chapter 2): This objective was to analyze whether stress during gestation in the parental (F0) generation causes transgenerational changes in gene expression in placenta and embryonic cortex in the offspring of the F3 generation.

Experiment 2 (Chapter 3): This objective was to analyze whether gestational stress alters miRNA expression in placenta and embryonic cortex in the F1, F2, and F3 generations.

Experiment 3 (Chapter 4): To analyze whether observed changes in mRNA expression in multiple generations of animals are caused by altered DNA methylation patterns in response to prenatal stress.

In each experiment we compared the cumulative effects of stress to the non-cumulative effects in each generation.

Chapter 2: Transgenerational effects of gestational stress on mRNA expression

Introduction

It has been acknowledged for a long time that prenatal stress is linked to abnormal cognitive, behavioural and psychosocial outcomes in both animals and humans. Substantial evidence from human epidemiological studies has linked prenatal stress to an increased vulnerability for developing psychological or neurological problems in childhood and adulthood, such as attention deficit hyperactivity disorder, anxiety- and depression-related disorders, schizophrenia, and autism among others (reviewed in (Babenko et al 2015)). The effects of prenatal stress were demonstrated in animal studies with regards to abnormalities in learning and memory (Lemaire et al 2000, Modir et al 2014, Wu et al 2007), attention deficits (Lehmann et al 2000, Schneider et al 1999), play and locomotor behaviour (Barrett et al 2014, Coe et al 2003, Morley-Fletcher et al 2003), and depression- and anxiety-like behaviour (Abe et al 2007, Estanislau & Morato 2005, Fride & Weinstock 1988, Vallee et al 1997b). These effects observed following prenatal stress are most likely mediated by the effects of stress on the fetal brain (Charil 2010). For example, it was shown that alteration in depression- and anxiety-like behaviour might be caused by reduced postnatal neurogenesis in the hippocampus (Coe et al 2003, Lemaire et al 2000, Rayen et al 2011). Most animal research has focused on the maternal hypothalamic-pituitary-adrenal (HPA) axis, however it appears that there is a weak association between maternal stress or anxiety during pregnancy and elevated maternal cortisol levels, suggesting that other mechanisms may be responsible for increased negative effects on offspring wellbeing [reviewed in (O'Donnell et al 2009)]. The main organ that protects the developing fetus from the negative effects of maternal stress is the placenta. Thus, the placenta may be an organ that contributes to the higher risks of adverse effects on the child. Moreover, a lot of attention has been paid recently to the involvement of epigenetic mechanisms in the programming of brain development and mental health and disease (reviewed in (Babenko et al 2015)). Epigenetics is the study of heritable changes in the gene expression profile of a cell that are not caused by changes

in the nucleotide sequence of the DNA. Altered epigenetic regulation may influence fetal endocrine programming and brain development that will be reflected by changes in mRNA expression in placenta and the brain across several generations. Therefore, we hypothesized that prenatal stress causes transgenerational changes in gene expression in developing brain that are mediated by the changes in gene expression in the placenta. In this experiment we utilized a rat model to assess mRNA expression in both placenta and fetal cortex in three generations. Parental generation (PO) of pregnant rats were exposed to stress from gestational day (G) 12 till G18 and three subsequent generations of offspring were assessed. Alteration of gene expression in the third generation offspring (F3) after parental stress exposure will indicate the possible involvement of epigenetic mechanisms in prenatal programming. Moreover, in this experiment we compared the effects of cumulative stress exposure (where pregnant rats were stressed during pregnancy across three generations) to single stress exposure (in parental generation only).

Materials and methods

Animals

Adult Long-Evans rats were housed in pairs in standard polycarbonate shoebox cages under a 12 h light/day cycle with lights on at 7:30AM. All procedures were performed in accordance with the Canadian Council for Animal Care guidelines and approved by the institutional Animal Welfare Committee.

Experimental groups

Three generations of animals (F1, F2, F3) were bred for this experiment. In each generation one group of animals was sacrificed on embryonic day E21 (both mothers and pups), whereas another group of animals was used for breeding of the subsequent generations (Fig. 2.1). The parental generation (F0) of animals was either stressed during gestation (S) or not stressed (N). In the F1 generation stressed animals were divided into two lines: “Cumulative Stress Exposure Line”, where adult animals were stressed during pregnancy in each subsequent

generation (F1 mothers: SS, F2 mothers: SSS) and "F0 Generation Stress Line", where animals were stressed only in parental generation (F1 mothers: SN, F2 mothers: SNN). Three generations of offspring from both stress exposure lines were used for the gene expression analysis (F1 pups: SN; F2 pups: SNN, SSN; F3 pups: SNNN, SSSN). For more details see Fig. 2.2. The sample size was n=6 for each of the experimental groups.

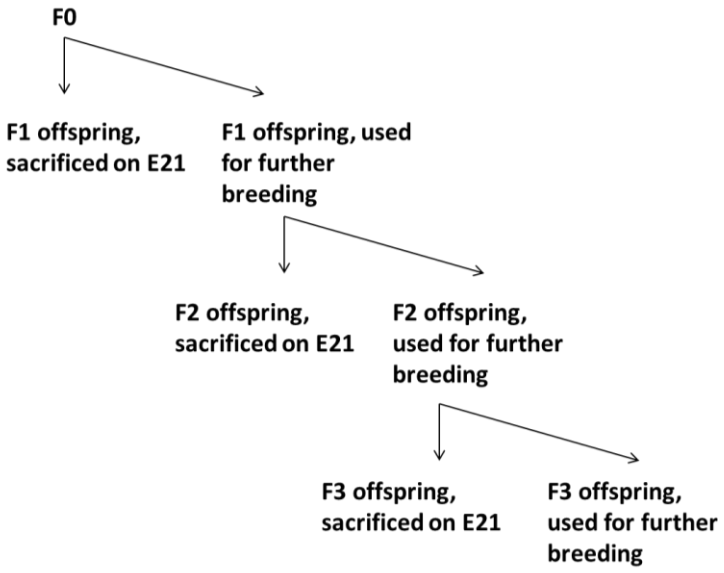


Figure 2.1. Breeding scheme. In each generation sub-groups animals were sacrificed on E21 (both mothers and pups), and other animals were used for breeding of subsequent generations.

Time course

Pregnant dams were stressed from G12 to G18. The start of the stress treatment coincides with the first phase of the epigenetic reprogramming that takes place in primordial germ cells (PGCs) when parental imprints are erased and totipotency is restored (Fig. 2.3).

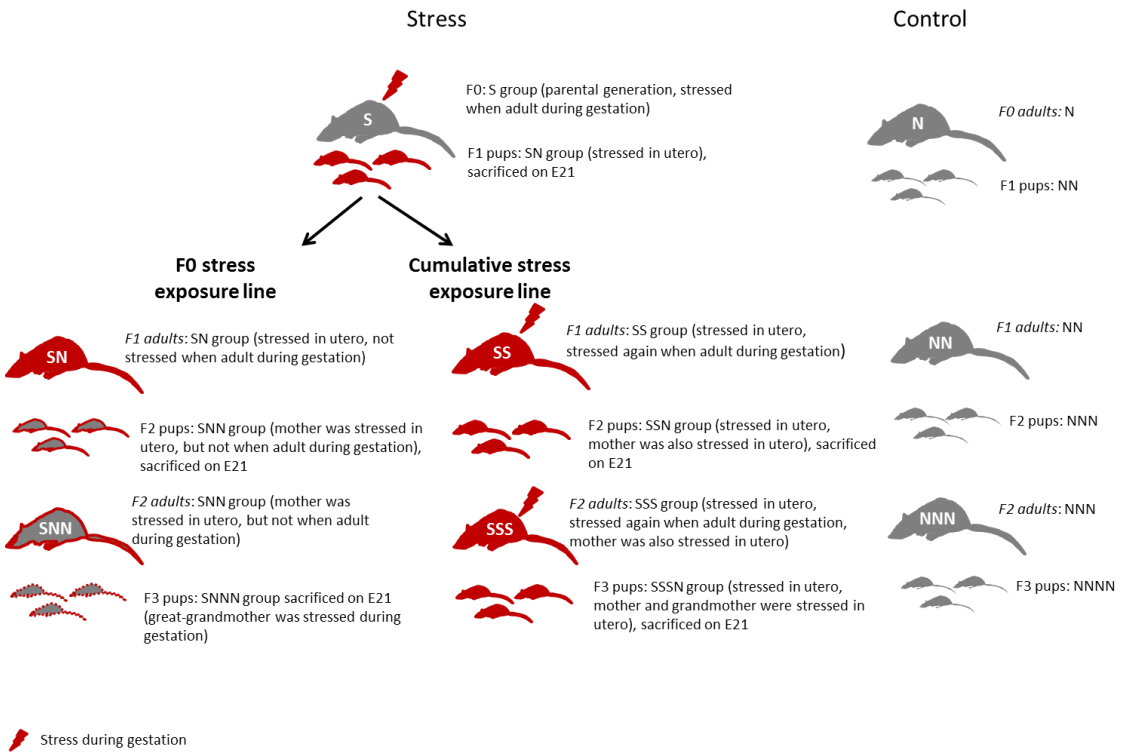


Figure 2.2. Experimental groups. The parental generation (F0) of animals was either stressed during gestation (S) or not stressed (N). In the F1 generation stressed animals were divided into two lines: "Cumulative Stress Exposure Line", where adult animals were stressed during pregnancy in each subsequent generation and "F0 Generation Stress Line", where animals were not stressed during pregnancy in subsequent generations.

Stress paradigm

The stress paradigm included 20 minutes of restraint and 5 minutes of swimming daily, applied in a semi-random alternation at 8:00 am and 4:00 pm from G12 till G18. Stress treatments were performed in a designated room other than the housing facility. This stress paradigm was previously established by our laboratory (Yao et al 2014).

Restraint stress. Animals were placed individually into transparent adjustable length Plexiglas tubes (diameter 8 cm, length 20 cm) for 20 minutes daily. The tube maintained the animal in a standing position. Small holes in the front of the tube allowed for ventilation.

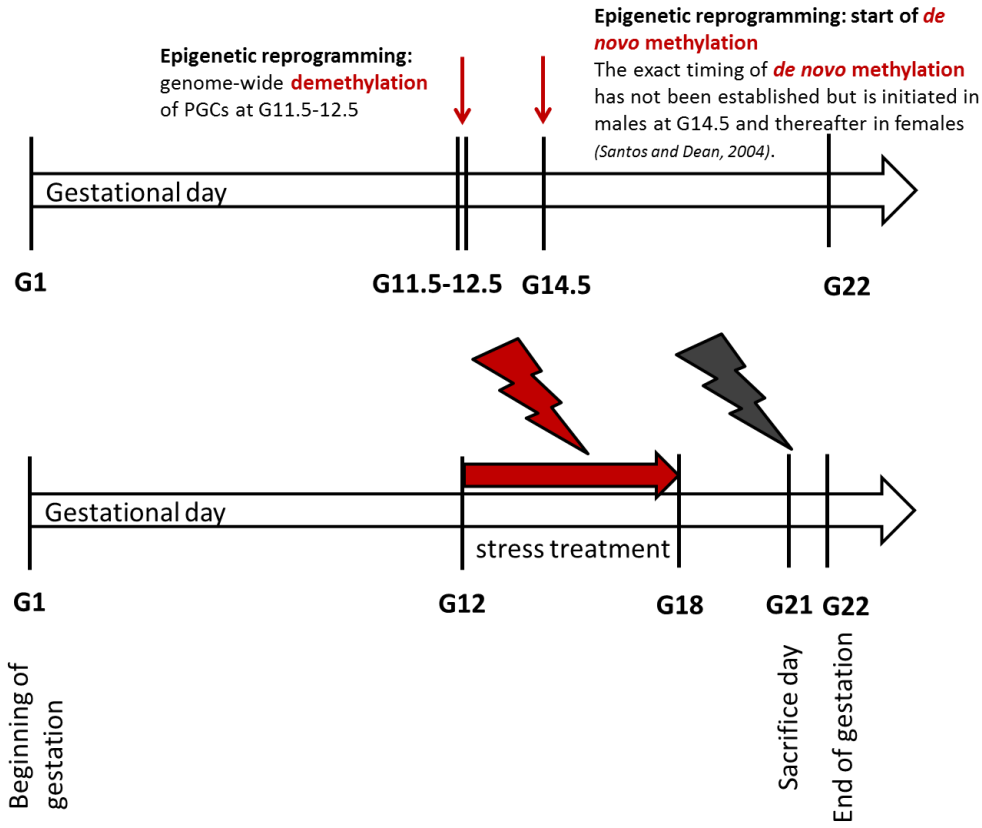


Figure 2.3. Time course of the experimental manipulations. The starting point of stress treatment coincides with the epigenetic reprogramming event when primordial germ cells (PGCs) undergo genome-wide demethylation at gestational days G11.5-G12.5. Another epigenetic reprogramming event occurs in the middle of the stress treatment on G14.5, when *de novo* methylation is initiated in males.

Swim stress. Rats were individually placed in a tub filled with room temperature water (~22 degrees Celsius) for five minutes. The water was deep enough so that neither the rat's feet nor its tail had contact with the bottom. After the 5 minutes dams were towel dried and returned to their home cage.

Tissue dissection and RNA extraction

Fetuses and placenta were extracted via caesarean section at G21. Pups were decapitated and whole brains were extracted. Embryonic brains were used for the dissection of cortex and cerebellum. Dissected cortex and placentas were used for RNA extractions. Total RNA isolation was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to

manufacturer's instructions. The samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and stored at -80°C. RNA was dissolved in RNase-free water (NEB, Ipswich, MA, USA).

mRNA profiling analysis

mRNA expression profiling was done, using Illumina Whole-Genome Expression Beadchips (catalogue numbers: 5665167043, 5665167040, 5665167032, 5665167042).

Library preparation

For this study, cRNA was created using the Ambion Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, CA), with an input of 500 ng of total RNA per sample. Briefly, oligo-dT primers were used to synthesize first-strand cDNA containing a phage T7 promoter sequence. The single-stranded cDNA was converted into a double-stranded DNA template via DNA polymerase. RNase H acted simultaneously to degrade RNA, and cDNA samples were purified in filter cartridges to remove excess RNA, primers, enzymes, and salts. The recovered cDNA was subjected to in vitro transcription using biotinylated UTPs. This step created the labeled and amplified cRNA. A final purification step removed unincorporated NTPs, salts, inorganic phosphates, and enzymes to prepare samples for hybridization.

Hybridization and detection

Illumina's direct hybridization assay kit was used to process samples according to the manufacturer's protocol (Illumina, San Diego, CA). Briefly, 750 ng from each cRNA sample was hybridized to the Illumina Rat-Ref-12 Whole Genome Expression BeadChip arrays overnight (each RatRef-12 BeadChip contains 22,523 probes per array targeting all known genes and known alternative splice variants). Afterward, a10-minute incubation with the supplied wash buffer at 55°C preceded a 5-minute room-temperature wash. The arrays were incubated in 100% ethanol for 10 minutes. A second room temperature wash for two minutes with gentle shaking completed this high stringency wash step. The arrays were blocked with buffer for 10 minutes and washed before a 10-minute probing with streptavidin-Cy3 (1:1000). After a five-minute wash at room

temperature, BeadChips were dried and imaged. Six controls were also built into the Whole-Genome Gene Expression Direct Hybridization Assay system to cover the aspects of array experiments. These included controls for a biological specimen (14 probes for housekeeping controls), three controls for hybridization (six probes for Cy3-labeled hybridization, four probes for low stringency hybridization, one probe for high stringency hybridization), signal generation (two probes for biotin control) and ~800 probes for negative controls on an eight-sample BeadChip. The arrays were scanned on the iScan platform (Illumina), and the data were normalized and scrutinized using Illumina BeadStudio software.

Data analysis and statistics

Data normalization and statistics was done using GenomeStudio software. Expression comparison between groups was completed using the Illumina custom model employing false discovery rate (FDR) correction and group comparison p-value thresholds of $p<0.05$. The detection p-value threshold of 0.01 was used to ensure that a given transcript was expressed above the background defined by negative control probes.

Gene functional classification and pathways analysis

Gene functional classification and pathway analysis was done, using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatic Resources 6.7 (Huang et al 2009a, Huang et al 2009b). The enrichment score (Fold Enrichment) used in the analysis is the geometric mean of all the enrichment p-values for each annotation term associated with the gene members in the group. It indicates the overall enrichment of gene groups. An enrichment score of 1.3 is equivalent to the non-log scale of 0.05.

Results

Maternal body weight gain during gestation

The average body weight of the mothers at the baseline was 342 ± 27 g and was not significantly different between the groups ($p>0.05$) (Fig.2.4). The average body weight gain

during gestation was 44.1 ± 6.5 g at G11, 87.9 ± 10.1 g at G18, 139.3 ± 14.1 at G21 was also not significantly different between the groups.

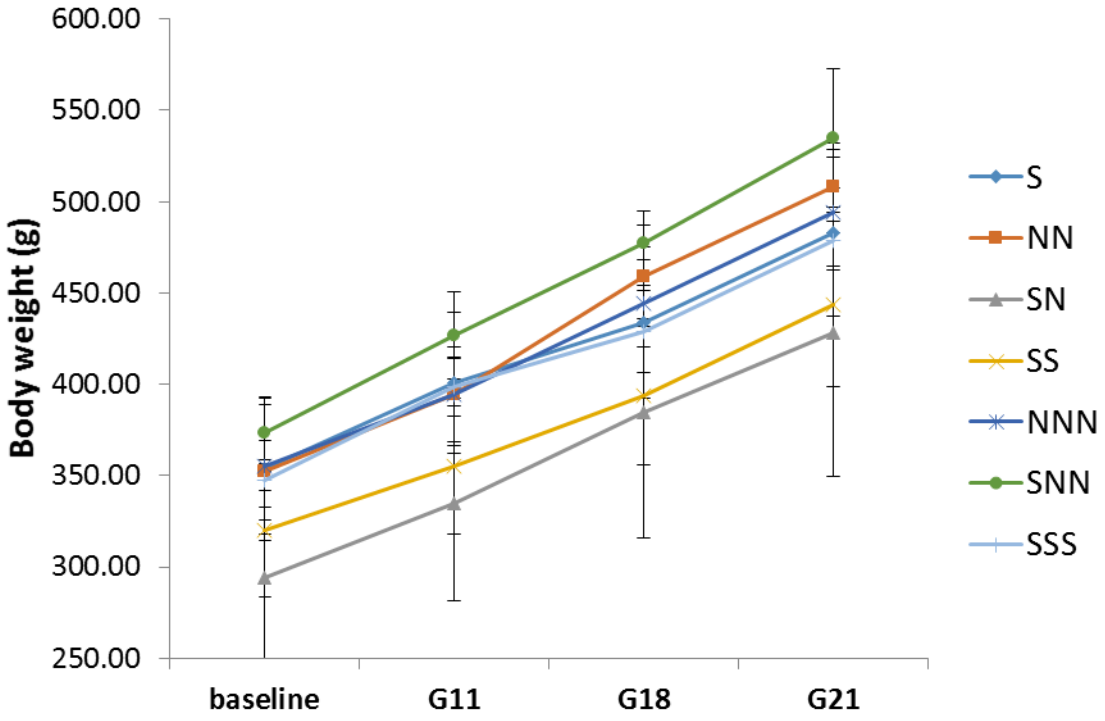


Figure 2.4. Average body weight of the pregnant dams during gestation (means ± standard deviation; g) as calculated from 4 animals per group.

Maternal adrenal glands weight and brain weight

Maternal adrenal glands weight and brain weight was not significantly different between groups in three generations (Fig. 2.5, 2.6).

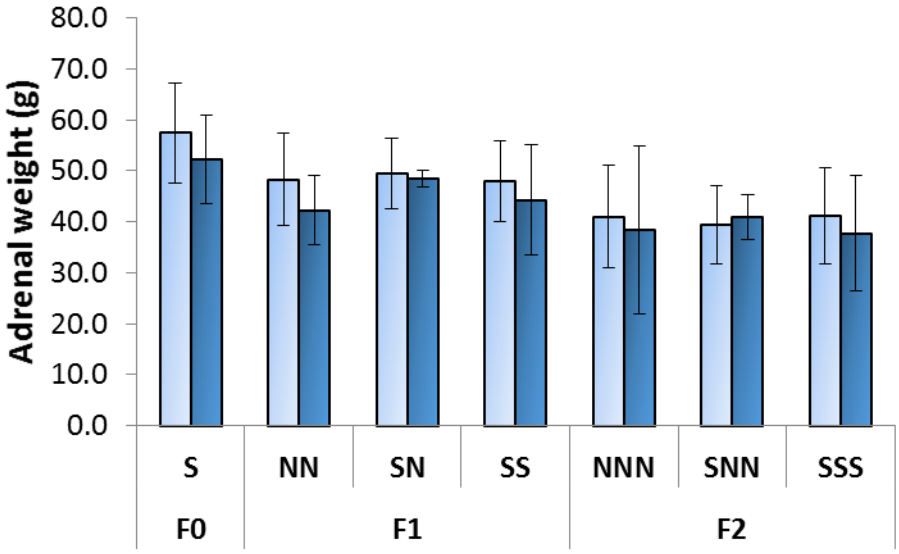


Figure 2.5. Weight of left (light blue) and right (dark blue) adrenal glands in three (F0, F1, F2) generations of stressed mothers (mean ± standard deviation; g)

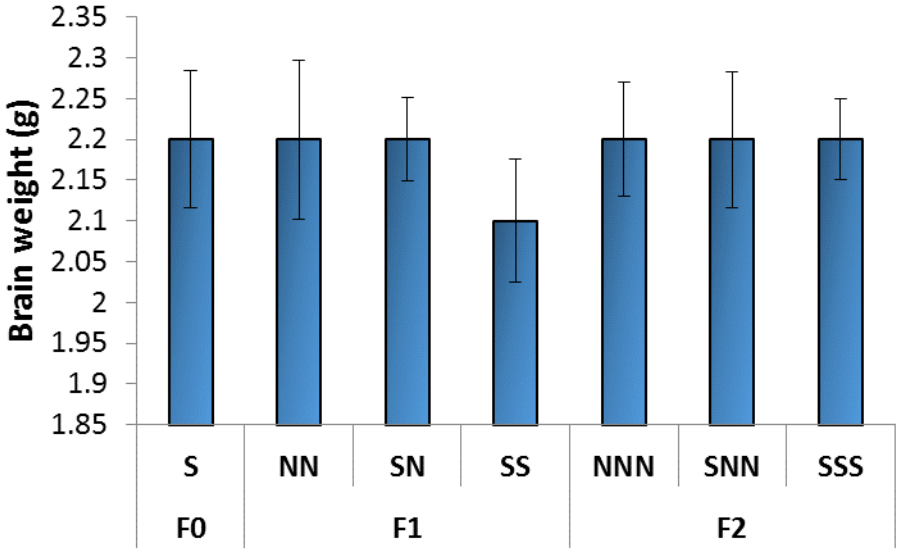


Figure 2.6. Maternal brain weight in three (F0, F1, F2) generations (mean ± standard deviation; g)

Litter size and sex distribution

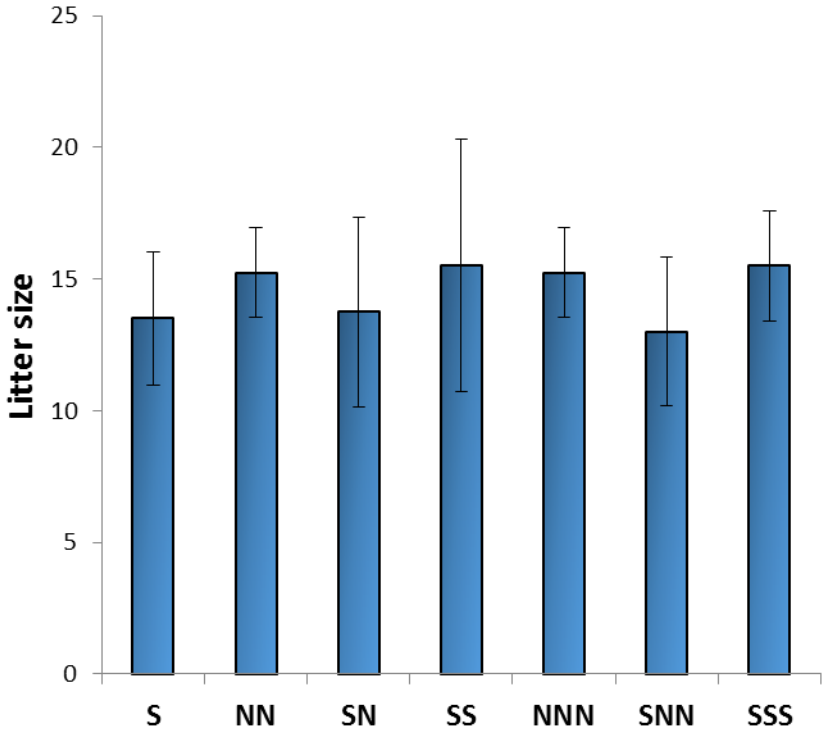


Figure 2.7. Litter size in three generations (mean ± standard deviation; pups per litter)

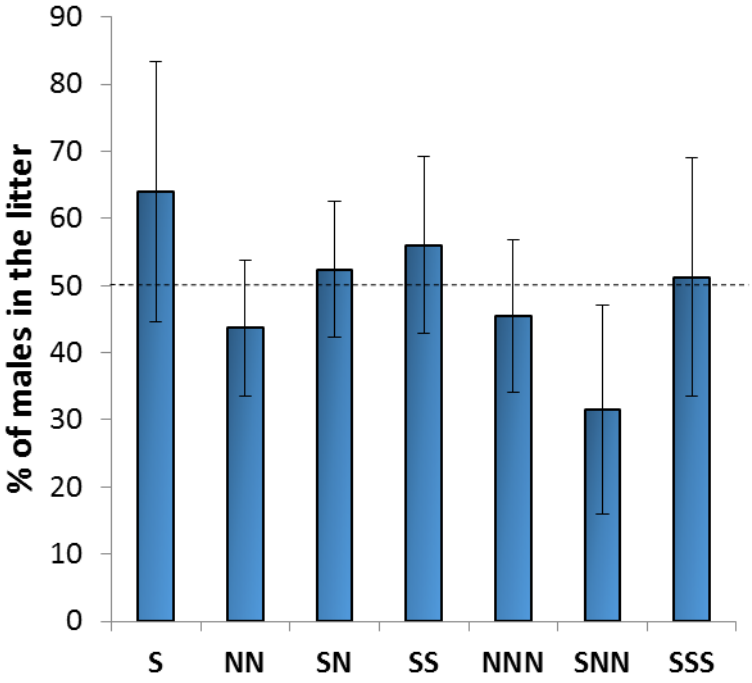


Figure 2.8. Sex distribution in the litter in three generations, shown in % males in the litter (mean ± standard deviation)

P1 pup weight

The offspring weight at E21 (sacrifice day) was not measured, but to verify whether prenatal stress had an effect on the offspring weight we assessed pups on postnatal day 1 (P1) in a separate cohort of animals subjected to the same stress paradigm. Our results showed that P1 weight of F1 prenatally stressed offspring (SN) was not significantly different from their respective controls (NN), whereas F2 and F3 offspring from F0-generation stress exposure lineage (SNN, SNNN) were significantly heavier than their respective controls (NNN, NNNN). In the cumulative stress exposure line only weight of the F2 offspring (SSN) was affected by prenatal stress (Fig. 2.9).

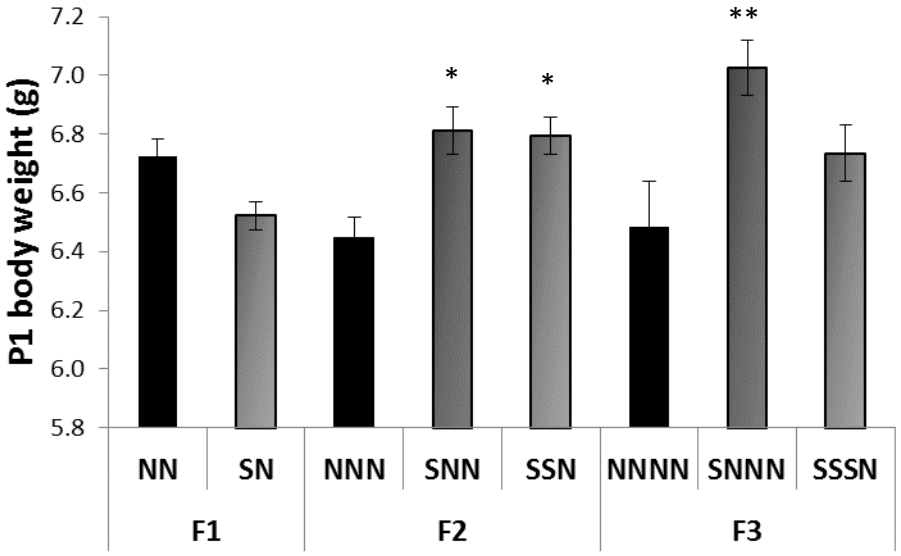


Figure 2.9. Offspring's body weight at postnatal day 1 (mean ± standard error; g). Asterisks represent statistical significance between exposure group and their respective controls (*$p<0.05$; **$p<0.01$).

mRNA microarray analysis

We analyzed the mRNA expression pattern in three generations of prenatally stressed animals from both cumulative and single-generation stress lines in comparison to non-stressed controls.

Microarray data analysis showed that expression of only three genes was significantly altered ($p < 0.05$) in F1 generation offspring (SN) in E21 cortex (Fig. 2.10, A) and two genes in placenta (Fig.2.10, B). Expression of 1,424 genes (6,3% of the total number of genes checked) was altered in cumulative stress exposure group in cortex in F2 generation (SSN) and 2,379 genes (10,6% of the total number of genes) in F3 generation (SSSN). Similar results were observed in placenta in cumulative stress exposure line (SSN: 1,526 genes (6.8% of the total number of genes); SSSN: 2,558 genes (11.4% of the total number of genes)).

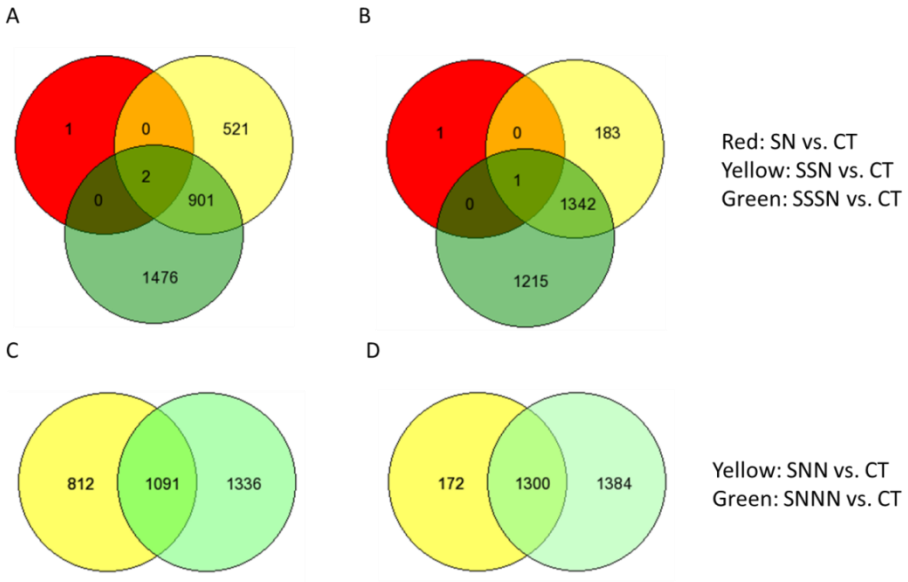


Figure 2.10. Venn diagrams showing the number of genes differentially expressed in the placenta (B, D) and G21 cortex (A, C) in three generations of prenatally stressed animals from both cumulative (A, B) and single-generation stress exposure lines (C, D). Sample size was n=6.

Maternal exposure to stress during gestation in parental generation (F0-stress exposure line: SNN, SNNN) resulted in significant changes in the gene expression in placenta and embryonic cortex of offspring from generations F2 and F3 (Fig. 2.10, C, D).

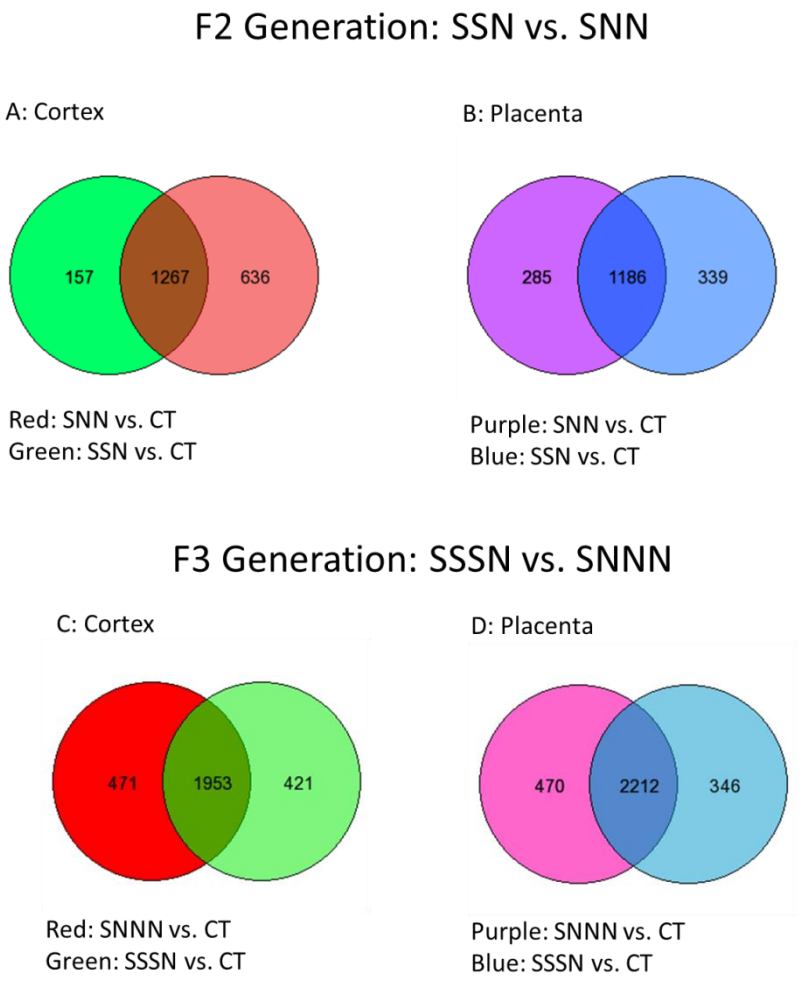


Figure 2.11. Venn diagrams showing the number of genes differentially expressed in cumulative stress exposure lineage (SSN, SSSN) vs. single-generation stress exposure lineage (SNN, SNNN) in G21 cortex (A, C) and placenta (B,D) in F2 and F3 generations. Sample size was n=6.

Some of the altered genes were common for cumulative and non-cumulative stress exposure lines in both F2 and F3 generations, whereas some of the genes were unique in each group (Fig. 2.11). Venn diagrams showing the number of up-regulated and down-regulated genes are shown in Fig. 2.12-2.13.

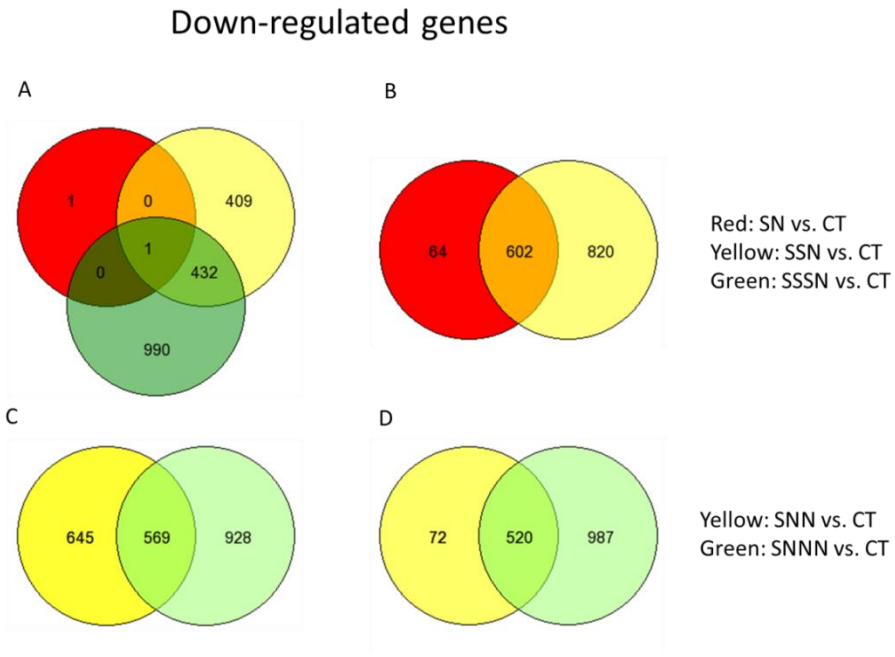


Figure 2.12. Venn diagrams showing the number of genes down-regulated in three generations of both cumulative stress exposure (A, B) and single-generation stress exposure (C, D) lines of animals in G21 cortex (A, C) and placenta (B, D). Sample size was n=6.

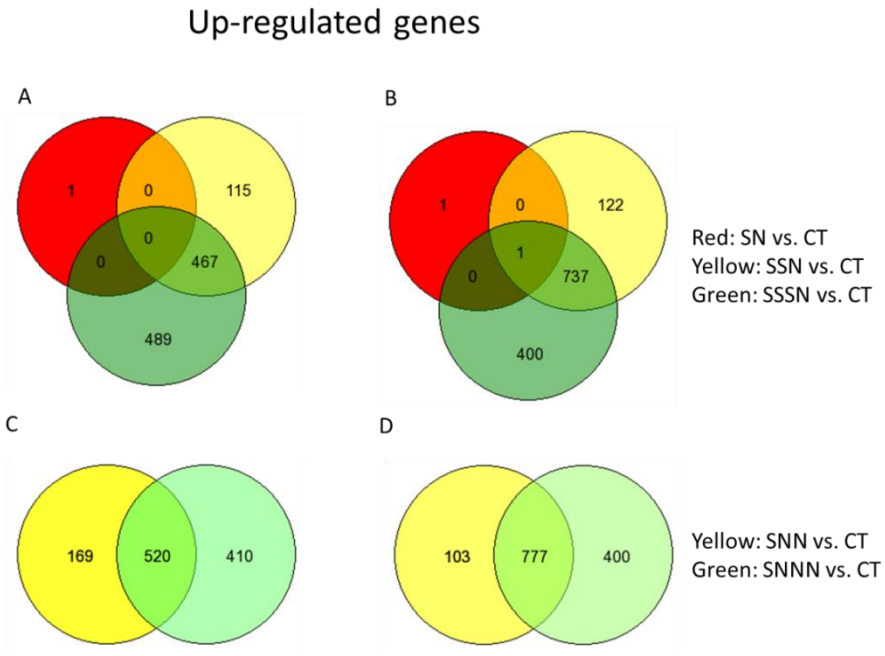


Figure 2.13. Venn diagrams showing the number of genes up-regulated in three generations of both cumulative stress exposure (A, B) and single-generation stress exposure (C, D) lines of animals in G21 cortex (A, C) and placenta (B, D). Sample size was n=6.

Gene functional classification and pathways analysis

To describe the possible role of the observed changes in gene expression we performed a gene functional classification and pathways analysis, using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatic Resources 6.7 (Huang et al 2009a, Huang et al 2009b). Since the main focus of this experiment was transgenerational epigenetic programming, we started with the analysis of the changes in SNNN group. Changes in the F3 generation may represent truly transgenerational epigenetic experience, whereas changes seen in the F1 and F2 generations are not necessarily heritable, since some of the modifications in F1 and F2 may be attributed to the direct effects of germline exposure.

The first criterion is the exposure of one generation to an (usually adverse) event that was never again repeated in subsequent generations and the number of generations that passed since that exposure (Crews, 2008 and Skinner, 2008). Accordingly, the second criterion is a minimum of two generations in the case of male exposure or three generations in the case of female exposure to claim truly transgenerational inheritance (Skinner, 2008).

Placenta

Gene classification clustering based on functional categories revealed 54 records with $p<0.05$ for the list of genes with altered expression in SNNN group in placenta (Supplemental materials, Table S1). Classification based on gene ontology resulted in 320 records with $p<0.05$, top three gene categories with the highest number of genes altered are shown in Figure 2.14, top 10 gene categories with the highest fold enrichment (FE) are shown in Supplemental materials (Table S2).

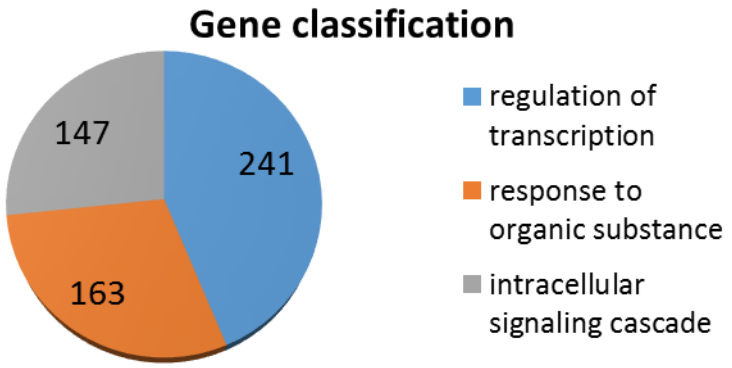


Figure 2.14. Gene classification based on gene ontology. Top three categories with the highest number of genes changed are shown. Numbers indicate the number of genes altered in each of the categories.

Two gene categories in the functional annotation chart are of particular importance for the embryonic development, including 47 genes from the category of "*In utero* embryonic development" (FE=1.6, p≤0.001) and 27 genes from the category "Female pregnancy" category (FE=2.1, p≤0.001) (see Tables 2.1, 2.2).

Table 2.1. List of genes from the "*In utero* embryonic development" category

GENE NAME	ENTREZ GENE ID
CCAAT/enhancer binding protein (C/EBP), alpha	24252
Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	64466
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	114490
EGL nine homolog 1 (C. elegans)	308913
GATA binding protein 6	29300
HOP homeobox	171160
PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae)	246216
SIN3 homolog A, transcription regulator (yeast)	363067
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	361825
Sp3 transcription factor	367846
adenosine deaminase	24165
amyloid beta (A4) precursor protein-binding, family A, member 3	83611
ankyrin repeat domain 11	365023
basic transcription factor 3; similar to basic transcription factor 3	500914
bone morphogenetic protein receptor, type IA	81507
cadherin 1	83502

calpain 2	29154
caudal type homeo box 2	66019
chromodomain helicase DNA binding protein 8	65027
cold shock domain protein A	83807
collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	365652
complement component (3b/4b) receptor 1-like	54243
cullin 3	301555
defender against cell death 1	192275
gap junction protein, alpha 1	24392
granulin	29143
hairy and enhancer of split 1 (Drosophila)	29577
hemoglobin alpha, adult chain 2	25632
integrin beta 1 (fibronectin receptor beta)	24511
keratin 19	360626
mitofusin 2	64476
mitogen activated protein kinase kinase 1	170851
myosin, heavy chain 9, non-muscle	25745
nibrin	85482
phosphatidylinositol transfer protein, beta	114561
plasminogen	85253
protein tyrosine phosphatase, receptor type, R	94202
resistance to inhibitors of cholinesterase 8 homolog A (C. elegans)	293614
serine peptidase inhibitor, Kunitz type 1	311331
similar to Bcl2-like 1 isoform 3; Bcl2-like 1	24888
tetratricopeptide repeat domain 27; baculoviral IAP repeat-containing 6	313876
tight junction protein 1	292994
transforming growth factor, beta 3	25717
tumor protein p53	24842
v-akt murine thymoma viral oncogene homolog 1	24185
v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	64188
zinc finger, AN1-type domain 5	293960

Table 2.2. List of genes for the "Female pregnancy" category

GENE NAME	ENTREZ GENE ID
Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	114490
FBJ osteosarcoma oncogene	314322
Kruppel-like factor 9	117560
Sp3 transcription factor	367846
acyl-CoA synthetase long-chain family member 4	113976

adiponectin receptor 2	312670
caldesmon 1	25687
carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	24240
carcinoembryonic antigen-related cell adhesion molecule 11	292668
complement component (3b/4b) receptor 1-like	54243
discoidin domain receptor tyrosine kinase 1	25678
fibulin 1	315191
ghrelin/obestatin prepropeptide	59301
granulin	29143
hydroxyprostaglandin dehydrogenase 15 (NAD)	79242
interleukin 11 receptor, alpha chain 1	245983
laminin, beta 1	298941
mucin 1, cell surface associated	24571
nicotinamide phosphoribosyltransferase	297508
plasminogen activator, urokinase	25619
prolactin family 7, subfamily a, member 3	64361
prolactin family 8, subfamily a, member 5	286889
prostaglandin-endoperoxide synthase 2	29527
steroid sulfatase	24800
transforming growth factor, beta 3	25717
trophoblast specific protein alpha	64509
wingless-type MMTV integration site family, member 4	84426

Analysis of the KEGG pathways revealed 40 chart records with FE≥1.2 and p-values of <0.1 (Table 2.3). Ten of these pathways play an important role in the development of the nervous system (Fig. 2.15, A), whereas 8 are involved in diseases, including several cancers and neurological disorders (Fig. 2.15, B).

Table 2.3. KEGG pathways for the list genes with altered expression in placenta in the SNNN group

Term	Count	%	P-Value	Fold Enrichment
Ribosome	43	1.7	0.000	3.8
Spliceosome	36	1.4	0.000	2.2
Lysosome	33	1.3	0.000	2.1
Cell cycle	33	1.3	0.000	1.9
Amino sugar and nucleotide sugar metabolism	15	0.6	0.001	2.5
Small cell lung cancer	23	0.9	0.001	2
Ubiquitin mediated proteolysis	31	1.2	0.001	1.8

p53 signaling pathway	19	0.8	0.003	2.1
Oocyte meiosis	26	1	0.007	1.7
Purine metabolism	33	1.3	0.009	1.6
Nucleotide excision repair	13	0.5	0.010	2.2
Insulin signaling pathway	29	1.1	0.010	1.6
Glutathione metabolism	14	0.6	0.014	2
Proteasome	14	0.6	0.014	2
Other glycan degradation	7	0.3	0.015	3.2
Neurotrophin signaling pathway	27	1.1	0.019	1.6
Pyrimidine metabolism	21	0.8	0.022	1.7
Fatty acid metabolism	12	0.5	0.022	2.1
MAPK signaling pathway	49	1.9	0.024	1.3
Prostate cancer	20	0.8	0.033	1.6
Pyruvate metabolism	11	0.4	0.033	2.1
Endometrial cancer	13	0.5	0.039	1.9
Valine, leucine and isoleucine degradation	12	0.5	0.042	1.9
Progesterone-mediated oocyte maturation	19	0.8	0.044	1.6
Acute myeloid leukemia	13	0.5	0.064	1.7
Glioma	14	0.6	0.065	1.7
Base excision repair	10	0.4	0.066	1.9
Long-term potentiation	15	0.6	0.066	1.6
Focal adhesion	35	1.4	0.076	1.3
Gap junction	17	0.7	0.079	1.5
Colorectal cancer	17	0.7	0.079	1.5
Chronic myeloid leukemia	16	0.6	0.080	1.6
Pancreatic cancer	15	0.6	0.081	1.6
Basal transcription factors	9	0.4	0.083	1.9
Pathways in cancer	53	2.1	0.087	1.2
Prion diseases	9	0.4	0.095	1.9
DNA replication	9	0.4	0.095	1.9
Lysine degradation	10	0.4	0.098	1.8
Biosynthesis of unsaturated fatty acids	7	0.3	0.099	2.1
mTOR signaling pathway	12	0.5	0.100	1.7

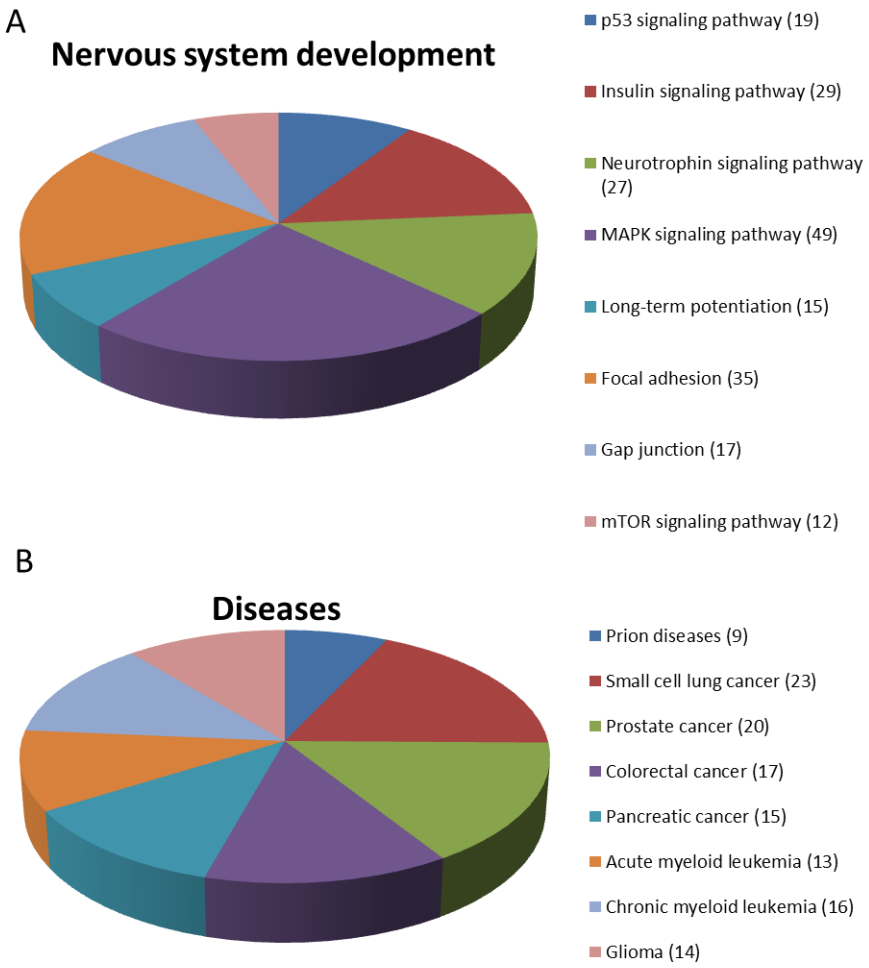


Figure 2.15. KEGG pathways. The number of genes altered in the pathway is indicated in brackets.

For more details on the pathways that play an important role in the nervous system development and diseases see Fig. 2.16, 2.17 and Fig. S1-S4 (Supplemental materials).

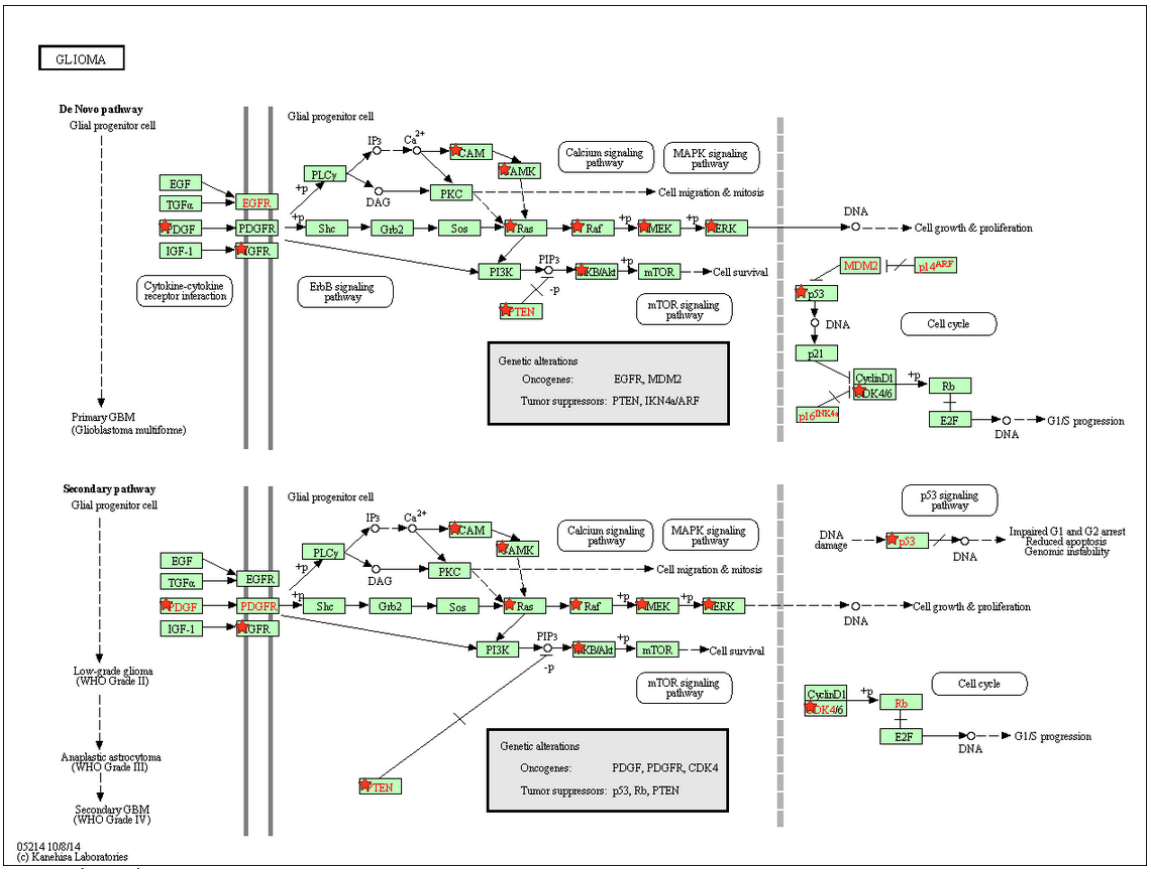


Figure 2.16. KEGG glioma pathway (Kanehisa & Goto 2000, Kanehisa et al 2014). Red stars indicate genes (14 out of 65) that were altered in response to stress.

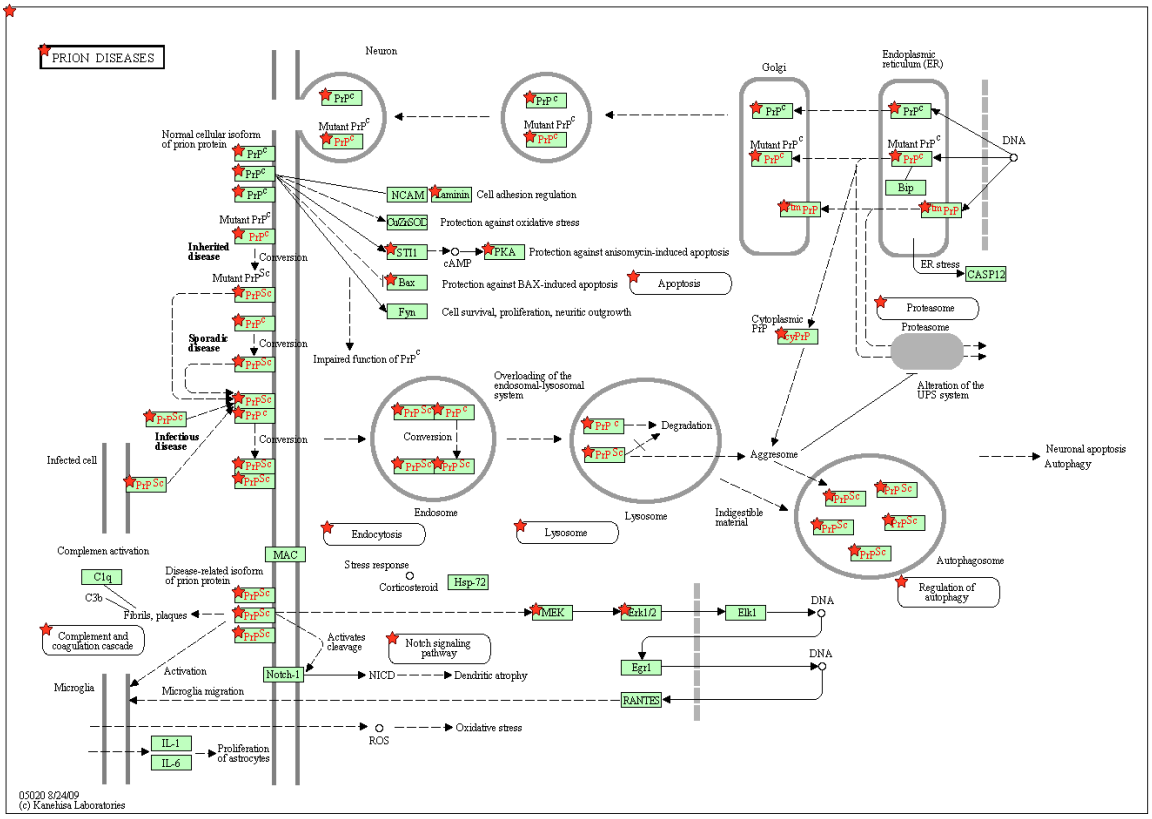


Figure 2.17. KEGG prion diseases pathway (Kanehisa & Goto 2000, Kanehisa et al 2014). Red stars indicate genes (9 out of 35) that were altered in response to stress.

Fetal cortex

Gene classification based on functional categories revealed 58 records with FE≥1.2 and p≤0.05 in the functional annotation chart (Supplemental materials, Table S3), whereas classification based on gene ontology resulted in 278 records with p≤0.05, 10 of which have a particular relevance to the development of nervous system (Table 2.4). The top three categories with the highest number of genes altered include "Regulation of transcription" (211 genes, FE=1.2, p=0.006), "Intracellular signaling cascade" (132 genes, FE=1.3, p=0.002), and "Protein localization" (127 genes, FE=1.7, p≤0.001) (Fig. 2.18).

Table 2.4. Functional annotation clustering based on gene ontology relevant to nervous system development (**Count** - number of genes that are altered in the category; **%** - percentage of total genes in the category**; Fold Enrichment** - fraction of genes in the category out of the total number of genes changed divided by total fraction of gene in category existing in the entire rat genome).

Term	Count	%	P-Value	Fold Enrichment
regulation of neurogenesis	35	1.5	0.014	1.5
regulation of neuron projection development	22	1	0.001	2.1
neuron projection development	46	2	0.005	1.5
learning or memory	26	1.1	0.006	1.8
neuron projection morphogenesis	37	1.6	0.009	1.5
learning	16	0.7	0.012	2
regulation of synaptic plasticity	17	0.7	0.016	1.9
dendrite development	11	0.5	0.021	2.2
regulation of nervous system development	36	1.6	0.032	1.4
neuron development	51	2.2	0.036	1.3

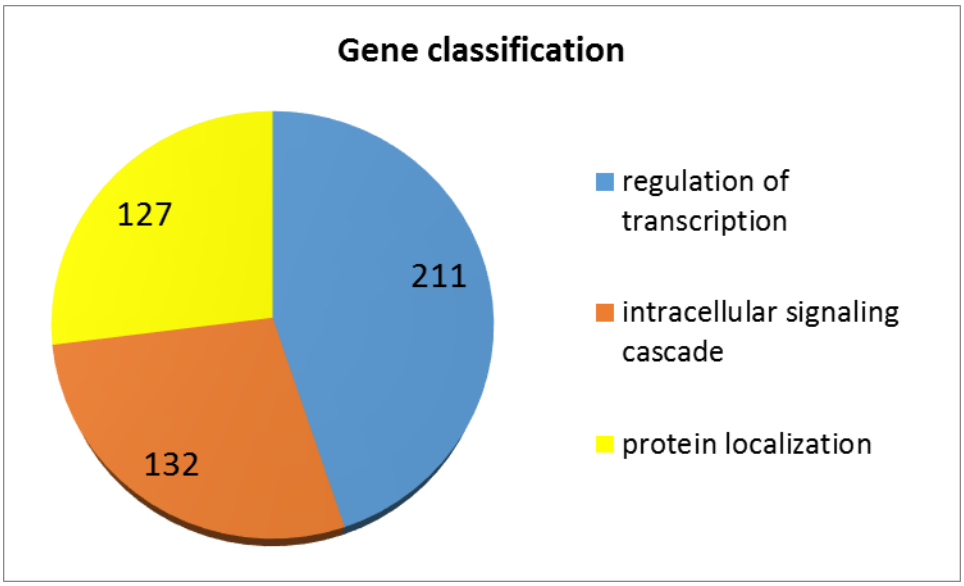


Figure 2.18. Gene classification based on gene ontology. Shown are the top three categories of genes with the highest numbers of genes altered. Numbers indicate the number of genes altered in each of the categories.

Gene classification based on tissue specificity revealed that 385 genes (17% of all genes from the list) fall into the category "Brain", including 86 genes that belong to the category "Pituitary" and 90 genes from the "Hippocampus" category (Table 2.5).

Table 2.5. Functional Annotation Chart based on tissue specificity

Term	Count	%	P-Value	Fold Enrichment
Pituitary	86	3.8	0.000	2
Brain	385	17	0.000	1.2
Spinal cord	71	3.1	0.000	1.7
Hippocampus	90	4	0.000	1.6
Heart	137	6	0.000	1.3
Prostate	165	7.3	0.001	1.3

The results of KEGG pathways analysis revealed the most significant changes (p-value<0.1) in four biological pathways, including Ribosome (FE=3.6, p≤0.0001), Spliceosome (FE=2.3, p≤0.0001), Ubiquitin mediated proteolysis (FE=1.9, p-value≤0.001), and Cell cycle (FE=1.9, p-value≤0.001) pathways (Table 2.6, Fig. 2.19).

Table 2.6. KEGG pathways for the list of genes with altered expression in the SNNN group

Term	Count	%	P-Value	Fold Enrichment
Ribosome	35	1.5	0.000	3.6
Spliceosome	33	1.5	0.000	2.3
Ubiquitin mediated proteolysis	29	1.3	0.001	1.9
Cell cycle	28	1.2	0.001	1.9
Base excision repair	12	0.5	0.004	2.7
Citrate cycle (TCA cycle)	10	0.4	0.006	2.8
Axon guidance	26	1.1	0.007	1.7
Amino sugar and nucleotide sugar metabolism	12	0.5	0.010	2.4
Endocytosis	36	1.6	0.011	1.5
Glutathione metabolism	13	0.6	0.012	2.2
Tight junction	25	1.1	0.017	1.6
Focal adhesion	34	1.5	0.020	1.5
Long-term potentiation	15	0.7	0.023	1.9
Glioma	14	0.6	0.024	1.9
ECM-receptor interaction	17	0.7	0.026	1.8
Fructose and mannose metabolism	9	0.4	0.029	2.4
SNARE interactions in vesicular transport	10	0.4	0.030	2.2
Prostate cancer	18	0.8	0.033	1.7
Oocyte meiosis	21	0.9	0.035	1.6

Aminoacyl-tRNA biosynthesis	10	0.4	0.041	2.1
Amyotrophic lateral sclerosis (ALS)	13	0.6	0.041	1.9
DNA replication	9	0.4	0.048	2.2
Gap junction	16	0.7	0.051	1.7
Small cell lung cancer	16	0.7	0.061	1.6
Purine metabolism	26	1.1	0.061	1.4
MAPK signaling pathway	41	1.8	0.063	1.3
Neurotrophin signaling pathway	22	1	0.065	1.5
Proteasome	11	0.5	0.065	1.9
RNA degradation	12	0.5	0.068	1.8
Endometrial cancer	11	0.5	0.073	1.8
Inositol phosphate metabolism	11	0.5	0.081	1.8
Non-small cell lung cancer	11	0.5	0.081	1.8
Pyruvate metabolism	9	0.4	0.083	1.9
Valine, leucine and isoleucine degradation	10	0.4	0.087	1.8
Insulin signaling pathway	22	1	0.096	1.4

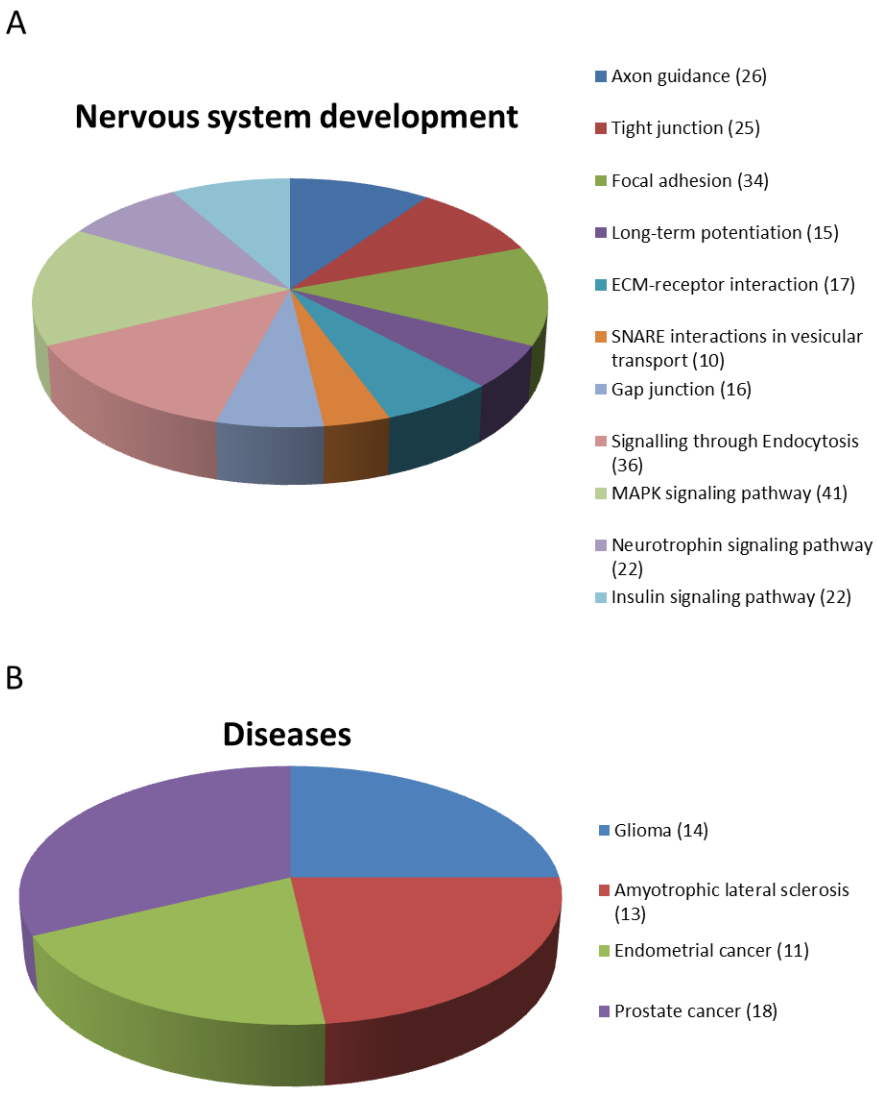


Figure 2.19. KEGG pathways important for nervous system development (A), and involved in diseases (B).

Most of the pathways important for the nervous system development were similarly altered in both placenta and cortex in SNNN group. Some of the pathways were unique for the cortex, including Axon guidance (26 genes, FE=1.7, $p \leq 0.01$), Signaling through endocytosis (36 genes, FE=1.5, $p \leq 0.05$), Tight junction (25 genes, FE=1.6, $p \leq 0.05$), ECM-receptor interaction (17 genes, FE=1.8, $p \leq 0.05$), and SNARE interactions in vesicular transport (10 genes, FE=2.2, $p \leq 0.05$). Among KEGG pathways were two involved in diseases that concern amyotrophic lateral

sclerosis and endometrial cancer, which were altered in E21 SNNN cortex, but not in placenta. Axon guidance pathway is shown on Fig. 2.20. For more details on other pathways that are of particular importance for the nervous system development and disease see Supplemental materials (Supplemental materials, Fig.S5 - S7).

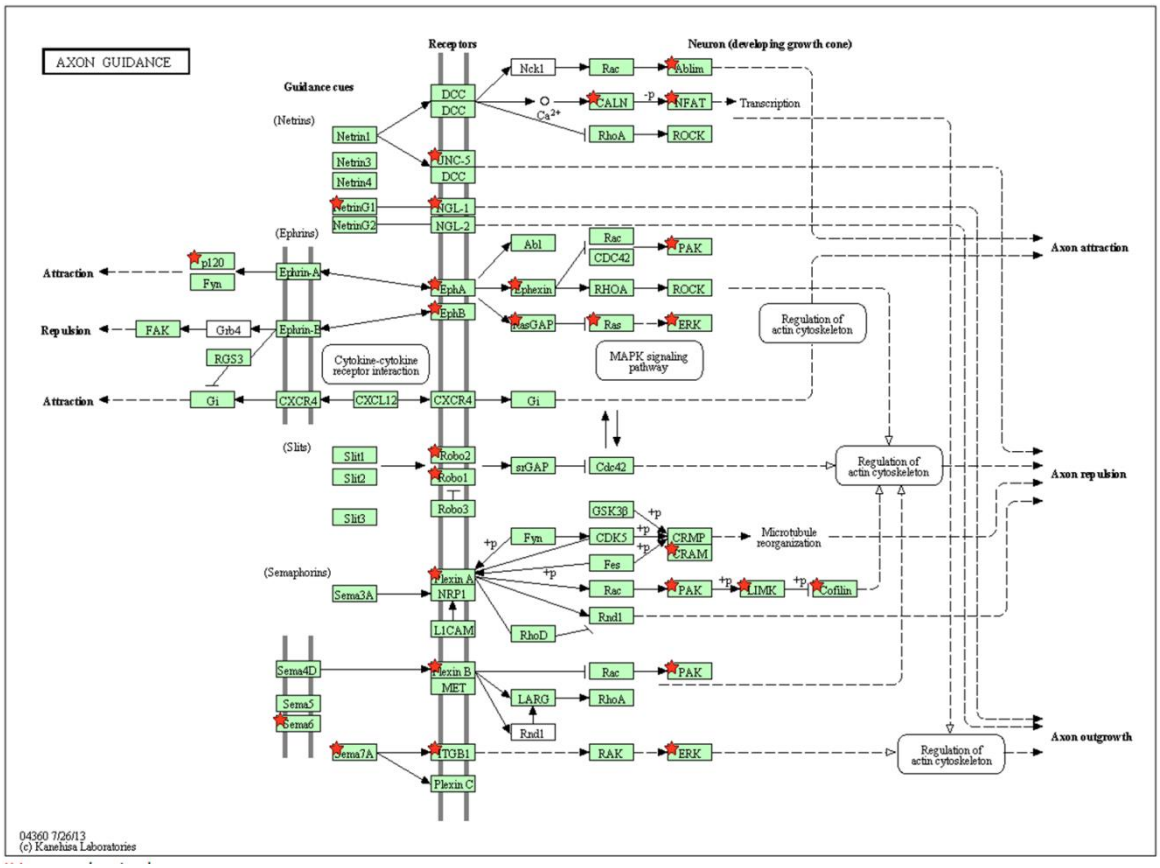


Figure 2.20. KEGG axon guidance pathway (Kanehisa et al 2014). Red stars indicate the genes (26 out of 127) that were altered in response to stress.

Cumulative effects of stress exposure on F3 generation offspring (SSSN vs. SNNN)

We analyzed changes in the KEGG pathways in SSSN group to check whether cumulative stress exposure caused any alterations that were unique for the SSSN group in comparison to SNNN. Our results showed that prenatal stress exposure caused similar changes in cumulative stress lineage. For the summary of the number of genes altered in different pathways in placenta and cortex in SNNN vs SSSN generations see Table 2.7.

Table 2.7. Comparison of the KEGG pathways in SNNN vs. SSSN groups

KEGG pathway	number of genes			
	placenta		cortex	
	SNNN	SSSN	SNNN	SSSN
Ribosome	43	39	35	33
Spliceosome	36	38	33	31
Ubiquitin mediated proteolysis	31	27	29	25
Cell cycle	33	31	28	3
Citrate cycle (TCA cycle)	X	9	10	9
Glutathione metabolism	14	12	13	13
Oocyte meiosis	26	25	21	24
Aminoacyl-tRNA biosynthesis	X	X	10	12
Prostate cancer	X	X	18	20
Small cell lung cancer	X	X	16	23
Proteasome	14	X	11	11
RNA degradation	X	14	12	13
Progesterone-mediated oocyte maturation	19	X	X	19
Fc gamma R-mediated phagocytosis	X	X	X	18
Fatty acid metabolism	12	X	X	9
Other glycan degradation	7	7	X	5
Colorectal cancer	X	X	X	17
Pancreatic cancer	X	X	X	15
Pathways in cancer	X	X	X	53
Base excision repair	10	X	12	X
Amino sugar and nucleotide sugar metabolism	15	14	12	X
Fructose and mannose metabolism	X	X	9	X
DNA replication	9	9	9	X
Purine metabolism	33	X	26	X
Inositol phosphate metabolism	X	X	11	X
Pyruvate metabolism	11	11	9	X
Valine, leucine and isoleucine degradation	12	X	10	X
Non-small cell lung cancer	X	X	11	X
Lysosome	33	38	X	X
Nucleotide excision repair	13	12	X	X
Pyrimidine metabolism	21	19	X	X
Lysine degradation	10	X	X	X
Biosynthesis of unsaturated fatty acids	7	X	X	X
Oxidative phosphorylation	X	31	X	X
KEGG pathways important for the nervous system development and disease				
Long-term potentiation	15	X	15	18
Neurotrophin signaling pathway	27	X	22	23

Insulin signaling pathway	29	**X**	22	**X**
MAPK signaling pathway	49	**X**	41	42
p53 signaling pathway	19	18	**X**	13
Gap junction	17	17	16	17
GnRH signaling pathway	**X**	**X**	**X**	18
TGF-beta signaling pathway	**X**	**X**	**X**	**X**
Adherens junction	**X**	**X**	**X**	14
Vascular smooth muscle contraction	**X**	**X**	**X**	**X**
ECM-receptor interaction	**X**	**X**	17	16
Focal adhesion	35	**X**	34	22
Signaling through endocytosis	**X**	37	36	37
Axon guidance	**X**	**X**	26	26
Long-term depression	**X**	**X**	**X**	13
Tight junction	**X**	**X**	25	25
Wnt signaling pathway	**X**	**X**	**X**	**X**
mTOR signaling pathway	12	12	**X**	**X**
SNARE interactions in vesicular transport	**X**	**X**	10	**X**
Glioma	14	**X**	14	12
Huntington's disease	**X**	35	**X**	29
Amyotrophic lateral sclerosis	**X**	**X**	13	**X**
Prion diseases	9	10	**X**	**X**
Alzheimer's disease	**X**	**X**	**X**	**X**

Several KEGG pathways were activated in the SSSN group, but not in the placenta of SNNN group, including Citrate cycle, RNA degradation, Oxidative phosphorylation, Signaling through endocytosis, and Huntington's disease pathways.

In E21 cortex some of the pathways were activated in SSSN group, but not in SNNN, including pathways important for the nervous system development and diseases, such as GnRH signaling pathway, Long-term depression, Huntington's disease (Supplemental materials, Fig. S8 – S10).

Generation-specific effects of stress (F2 vs. F3 generation)

To check whether pathways important for the nervous system development, that were altered in the F3 generation are similar to those altered in previous generation, we compared

KEGG pathway analysis results in F2 vs F3 for both cumulative (SSN, SSSN) and non-cumulative (SNN, SNNN) stress lineages in placenta and cortex (for the summary see Table 2.8).

Table 2.8. Comparison of the number of genes altered in KEGG pathways important for nervous system development in F2 vs F3 generations.

KEGG pathway	number of genes							
	F2 generation				F3 generation			
	placenta		cortex		placenta		cortex	
	SNN	SSN	SNN	SSN	SNNN	SSSN	SNNN	SSSN
Long-term potentiation	13	11	15	13	15	X	15	18
Neurotrophin signaling pathway	15	X	X	15	27	X	22	23
Insulin signaling pathway	17	19	X	X	29	X	22	X
MAPK signaling pathway	29	30	41	27	49	X	41	42
p53 signaling pathway	10	X	X	X	19	18	X	13
Gap junction	14	12	X	11	17	17	16	17
GnRH signaling pathway	13	X	15	X	X	X	X	18
TGF-beta signaling pathway	12	X	X	X	X	X	X	X
Adherens junction	11	13	X	X	X	X	X	14
Vascular smooth muscle contraction	14	X	X	X	X	X	X	X
ECM-receptor interaction	X	12	X	X	X	X	17	16
Signaling through endocytosis	X	27	X	21	X	37	36	37
Axon guidance	X	X	19	17	X	X	26	26
Long-term depression	X	X	12	X	X	X	X	13
Tight junction	X	X	19	X	X	X	25	25
Wnt signaling pathway	X	X	19	X	X	X	X	X
Calcium signaling pathway	X	X	X	19	X	X	X	X
mTOR signaling pathway	X	X	X	X	12	12	X	X
SNARE interactions	X	X	X	X	X	X	10	X

The mTOR signaling pathway was altered in F3 SNNN group, but not in F2 SNN in placenta in non-cumulative stress lineage. In the cumulative stress lineage P-53 signaling pathway

was altered in addition to mTOR signaling pathway. Some of the changes were unique to the F2 generation, including GnRH (Fig. S10) and TGF-beta signaling (Fig. S11), Vascular smooth muscle contraction, and Adherens junction pathways were altered in the F2 SNN, but not in the F3 SNNN group in placenta (Table 2.8).

In E21 cortex Neurotrophin and Insulin signaling pathways, Gap junction, ECM-receptor interaction, Signaling through endocytosis pathways were altered in F3 SNNN group, but not in F2 SNN. Altered pathways, unique for the F3 SSSN group in cumulative stress exposure lineage included P-53, GnRH signaling, Adherens junctions, Long term depression, and Tight junction pathways.

Glucocorticoid receptor expression and signaling

Results of mRNA profiling showed that mRNA expression of glucocorticoid receptor (*Nr3c1*) was not altered in response to stress in placenta or E21 cortex. However, we decided to check whether there was an effect of prenatal stress on expression of glucocorticoid target genes and co-transcription factors (*Atf4, Cebpa, Cebpb, Creb1, Creb3, Creb3l4, Pou2f1, Pou2f2, Stat5a, Stat5b, Stat3*). Expression of *Atf4*, *Cebpa*, *Stat5b*, Stat3, and *Crebl1* was altered in different groups. *Atf4* was up-regulated in SNNN group only, whereas *Crebl1* was down-regulated in SSSN and SNNN groups. *Stat5b* was down-regulated in SSN and SSSN groups only, whereas *Stat3* and *Cebpa* were down-regulated in SNN, SSN, SNNN, and SSSN groups. Several transcription factors were altered among glucocorticoid receptor target genes, including *Klf9* and *Zfp36l1* (were up-regulated in SNN, SSN, SNNN, and SSSN groups), and *Tsc22d3* (was up-regulated in SNNN and SSSN groups only). Summary of the expression of the glucocorticoid target genes in the E21 embryonic cortex in SNNN group is shown below (Fig. 2.21).

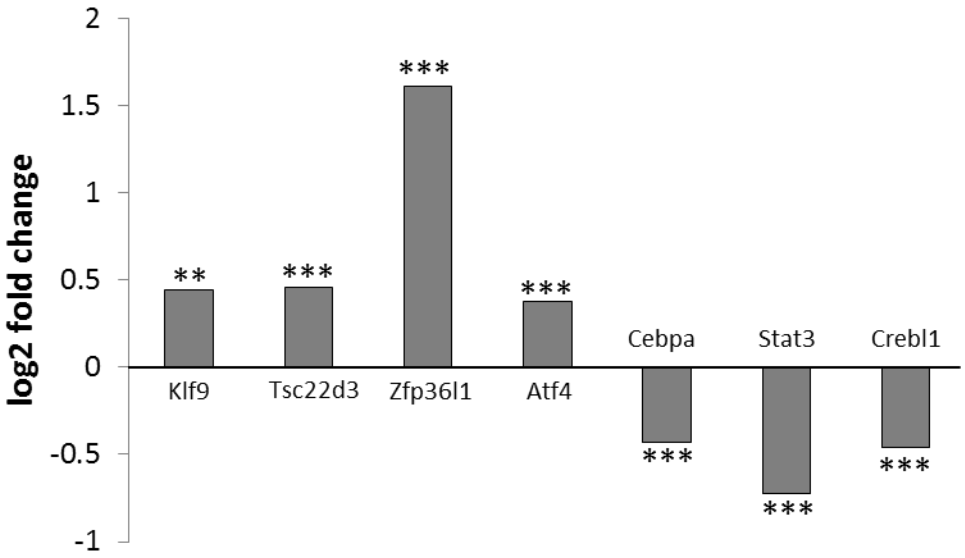


Figure 2.21. Expression of glucocorticoid target genes in E21 cortex in SNNN group. Data are presented as log2 fold change from control (**p≤0.01; ***p≤0.001; n=6).

Effects of stress on placental glucocorticoid barrier

Our mRNA profiling results showed that there were no significant differences between SSN and SNN group in cortex in F2 generation. In placenta only two genes were found to be differentially expressed between single generation stress exposure group and cumulative stress exposure group, including *Hsd11b1* and LOC293871. *Hsd11b1* together with *Hsd11b2* isoform and multidrug resistance P-glycoproteins (*Abcb1a and Abcb1b*) are important members of glucocorticoid barrier that protects fetus from the excessive levels of maternal glucocorticoids (Mark et al 2009). Therefore we checked whether *Hsd11b1, Abcb1a and Abcb1b* were altered in other comparisons in mRNA profiling results. Our analysis revealed that *Hsd11b1,* but not *Hsd11b2* was up-regulated in placenta in both F2 and F3 generations. Expression of *Abcb1b* was decreased in placenta in SSN group in F2 generation only. No changes in expression of other members of the placental glucocorticoid barrier were found in either the placenta or E21 cortex.

Effects of stress on prostaglandin synthesis

Since *Hsd11b1* is involved in prostaglandin synthesis we checked if the expression of other members of this pathway were altered in response to stress in mRNA profiling results. Our

analysis revealed that mRNA expression of the most members of the pathway was altered in both cumulative and non-cumulative stress exposure lineages in F2 and F3 generations in placenta. Results for the F3 SNNN group are summarized in Fig. 2.22.

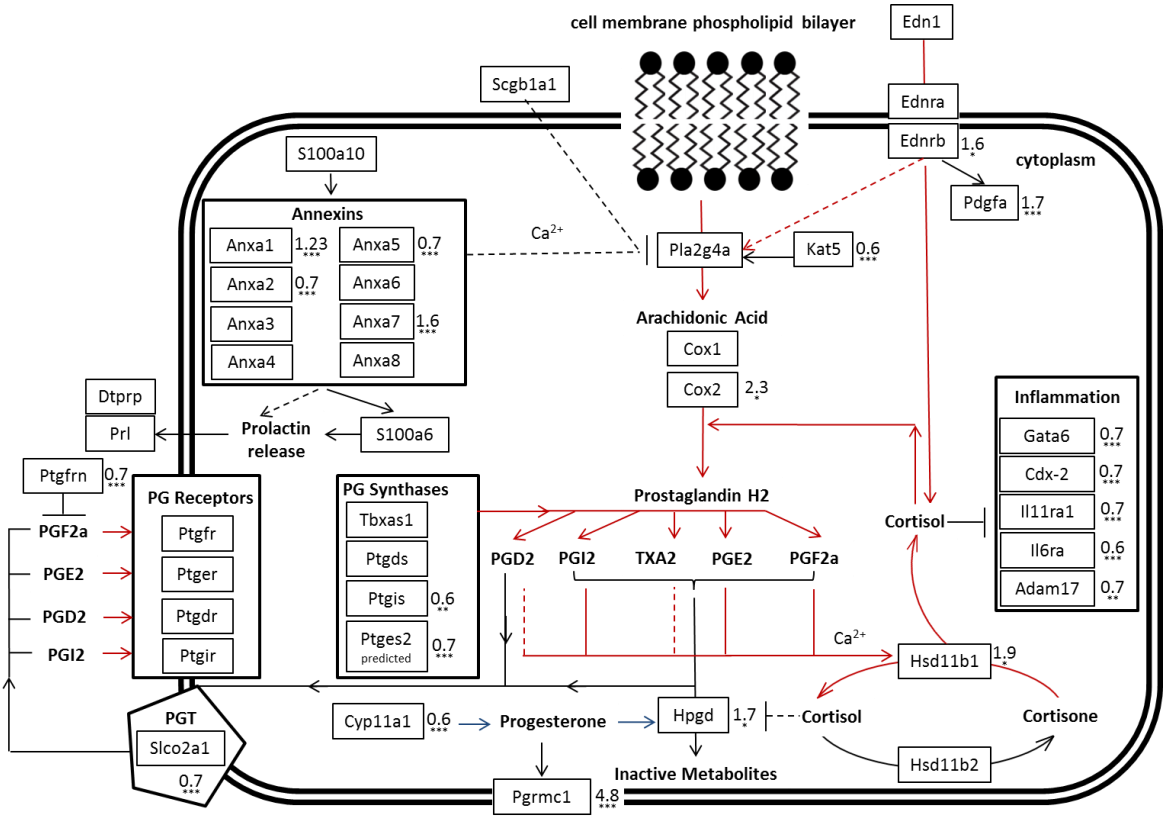


Figure 2.22. Prostaglandin synthesis and regulation pathway. Data for SNNN group. Numbers indicate fold change (SNNN vs. control), asterisks indicate statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001). Figure modified and redrawn from (Salomonis et al 2005) – "Analysis of pathways of uterine smooth muscle contraction. Prostaglandin synthesis" and from (Jabbour & Sales 2004) – "The cyclooxygenase (COX) and prostanoid biosynthetic and signaling pathways".

Summary of the significant changes (with p≤0.05) in the mRNA expression of the genes involved in the prostaglandin synthesis and regulation pathway is shown below (Table 2.9). Similar changes in the gene expression were observed in the pathway members in both stress lineages in F2 and F3 generations (Table 2.9).

Table 2.9. Changes in the mRNA expression of the members of prostaglandin synthesis and regulation pathway in placenta in F2 and F3 generations.

Gene	Fold change			
	F2 generation		F3 generation	
	SNN	SSN	SNNN	SSSN
Ednrb	X	1.4	1.6	1.5
Anxa1	2.0	2.0	1.2	2.3
Anxa2	0.7	0.7	0.7	0.6
Anxa5	0.7	0.7	0.7	0.6
Anxa7	1.3	1.3	1.6	1.5
Kat5	0.7	0.6	0.6	0.6
Pdgfa	1.3	1.3	1.7	1.6
Cox2	2.3	1.5	2.3	2.1
Ptgfrn	0.8	X	0.7	X
Slco2a1	X	X	0.7	0.7
Ptgis	X	X	0.6	0.7
Ptges2	X	X	0.7	0.7
Cyp11a1	0.6	0.7	0.6	0.6
Pgrmc1	2.3	2.3	4.8	4.5
Hpgd	1.9	1.6	1.7	X
Hsd11b1	X	1.5	1.9	1.6
Gata6	X	X	0.7	X
Cdx2	X	X	0.7	0.8
Il11ra1	0.7	0.7	0.7	0.7
Il6ra	X	0.8	0.6	0.7
Adam17	X	0.8	0.7	0.7

Effect of stress on expression of imprinted genes

To check whether prenatal stress had an influence on genomic imprinting, we analyzed the expression of imprinted genes in placenta and E21 cortex. Using the list of known imprinted genes from the Geneimprint database (http://www.geneimprint.com/) we assessed whether or not the expression of these genes was altered in our mRNA profiling results. The results showed that overall expression of 20 imprinted genes was altered in different group comparisons (Table 2.10, 2.11, Fig. 2.23).

Table 2.10. Imprinted genes with altered expression in F2 and/or F3 generation

Placenta				E21 Cortex			
SNN	**SSN**	**SNNN**	**SSSN**	**SNN**	**SSN**	**SNNN**	**SSSN**
Ampd3	*Ampd3*	*Ampd3*	*Ampd3*	*Ascl2*	*Cdkn1c*	*Cd81*	*Ascl2*
Cdkn1c	*Cdkn1c*	*Cdkn1c*	*Blcap*	*Cdkn1c*	*Snurf*	*Cdkn1c*	*Blcap*
Igf2	*Dcn*	*Dcn*	*Cdkn1c*	*Nnat*		*Gabra5*	*Cd81*
Ndn	*H19*	*Dio3*	*Dcn*	*Rasgrf1*		*Igf2*	*Cdkn1c*
Plagl1	*Igf2*	*Igf2*	*Igf2*	*Snurf*		*Tssc4*	*Gabra5*
Snrpn	*Ndn*	*Ndn*	*Ndn*				*Tssc4*
	Plagl1	*Plagl1*	*Plagl1*				
	Snrpn	*Pon2*	*Pon2*				
		Slc22a3	*Snrpn*				
		Snrpn					
		Snurf					
		Zrsr1					

Table 2.11. List of imprinted genes with altered mRNA expression in response to prenatal stress

gene	mRNA expression	expressed allele
Tssc4	decreased	maternal
Ampd3	increased	maternal
Cdkn1c	decreased	maternal
Dcn	increased	maternal
Pon2	increased	maternal
Cd81	decreased	maternal
Slc22a3	increased	maternal
H19	decreased	maternal
Ascl2	decreased	maternal
Nnat	decreased	paternal
Rasgrf1	decreased	paternal
Zrsr1	decreased	paternal
Igf2	decreased	paternal
Ndn	increased	paternal
Plagl1	increased	paternal
Snrpn	increased	paternal
Dio3	decreased	paternal
Snurf	increased	paternal
Blcap	decreased	isoform dependent
Gabra5	increased	Unknown

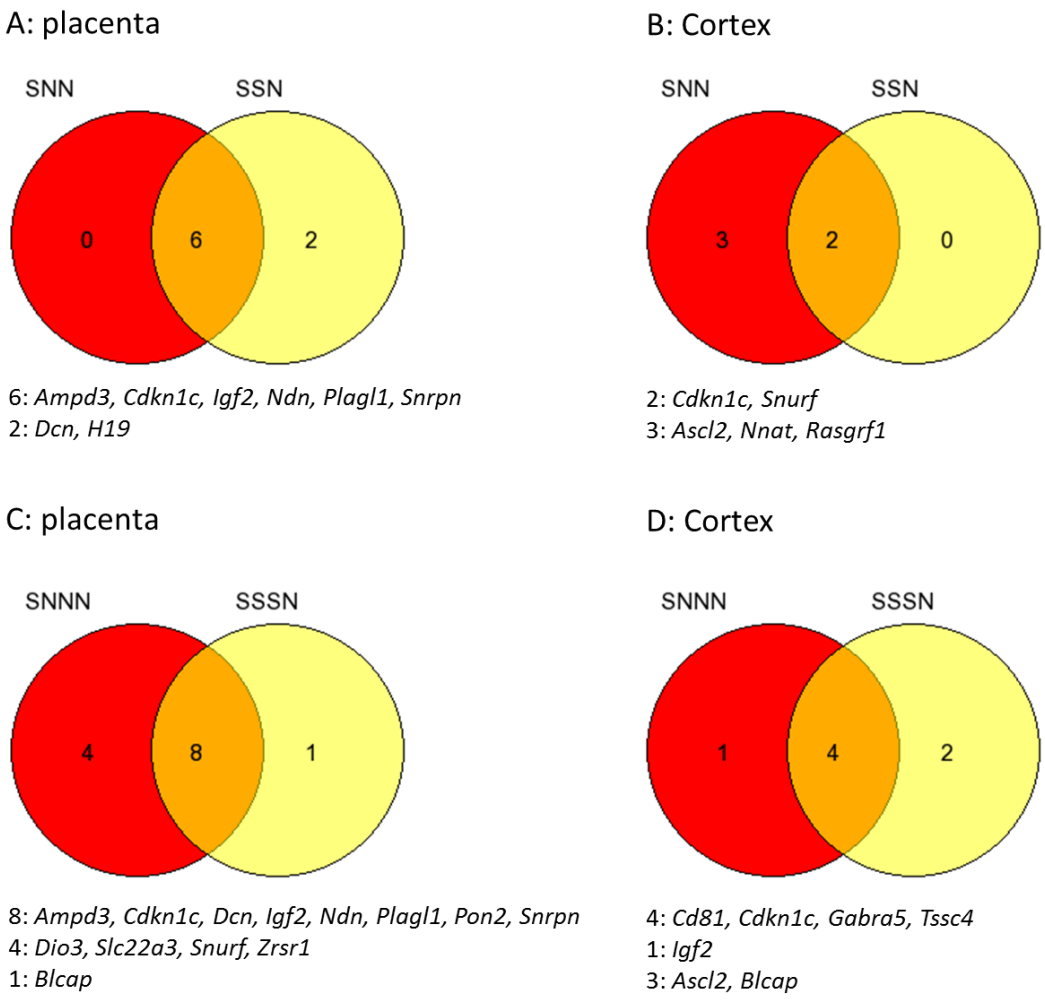


Figure 2.23. Venn diagrams showing imprinted genes that were differentially expressed in response to stress in placenta (A, C) and cortex (B, D) in cumulative (red) vs non-cumulative (yellow) stress exposure lineage in F2 (A, B) and F3 (C, D) generations. Sample size was n=6.

Expression of DNMTs

Since clusters of imprinted genes are known to be regulated by imprinting control regions (ICRs) that are characterized by DNA methylation of one allele, we checked whether the expression of DNA methyltransferases was altered in cortex or placenta. Our results showed that *Dnmt1* was down-regulated in SNNN group (FC=0.7, p≤0.001) in the cortex, whereas *Dnmt3a* was up-regulated in F2 generation SNN group (FC=1.9, p≤0.05), but not SSN. *Dnmt3a* was also up-regulated in F2 generation in placenta in both SNN (FC=1.4, p≤0.05) and SSN groups (FC=1.4, p≤0.001), but not in F3 generation.

Effects of stress on expression of neurotransmitter receptors and transporters in E21 cortex

Stress is known to influence the functioning neuroreceptors, such as dopamine receptors (*Drd1, Drd2, Drd3, Drd4, Drd5*) and serotonin receptors (*Htr1a, Htr1b, Htr1d, Htr1f, Htr2a, Htr2b, Htr2c, Htr3a, Htr3b, Htr4, Htr5a, Htr6, Htr7*). Our results showed that expression of *Drd2* was decreased in SNN, SSN, and SNNN group, whereas *Htr7* expression was increased in SNNN and SSSN groups in E21 cortex (Fig. 2.24). We also found that catechol-O-methyltransferase mRNA (*Comt*) that is physiologically important for dopamine metabolism in prefrontal cortex was decreased in cortex in SNNN and SSSN groups. Genes that encode other enzymes responsible for neurotransmitter synthesis and degradation, such as *Dbh, Ddc, Maoa, Th*, were not altered in the cortex.

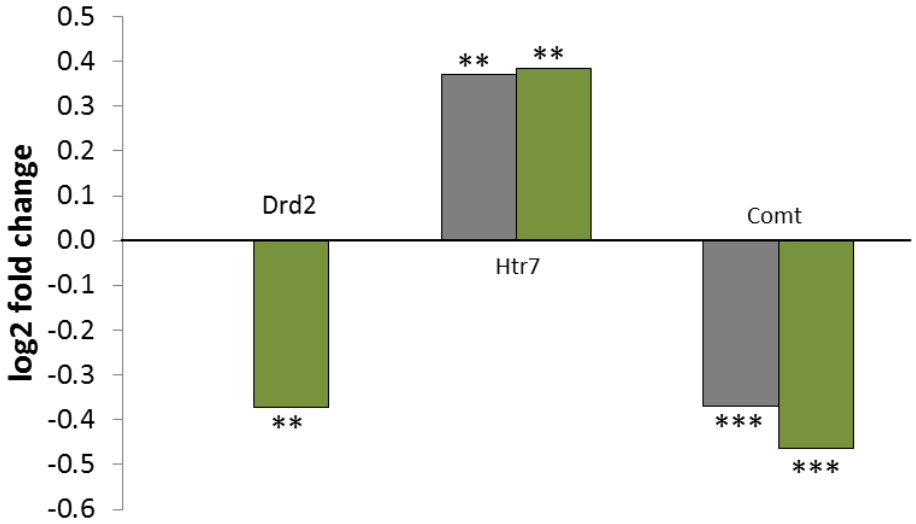


Figure 2.24. Neurotransmitter receptors expression and metabolism. mRNA expression in E21 cortex of F3 generation offspring in SNNN (green) and SSSN (grey) groups. Data are presented as log2 fold change from controls (**p≤0.01; ***p≤0.001; n=6).

Several neurotransmitter transporters (Slc6a family) were also found to be altered in addition to the observed changes in dopamine and serotonin receptors (Table 2.12, Fig. 2.25).

Table 2.12. Neurotransmitter transporters of the Slc6a family

gene	Log2 Fold change			
	F2 generation		F3 generation	
	SNN	SSN	SNNN	SSSN
Slc6a1	-0.6	-0.7	-0.6	-0.6
Slc6a13	-0.5	-0.5	-0.8	-0.6
Slc6a6	-0.5	-0.5	X	X
Slc6a9	-0.4	X	X	X
Slc6a20	X	X	-0.4	-0.4
Slc6a15	X	X	X	0.5

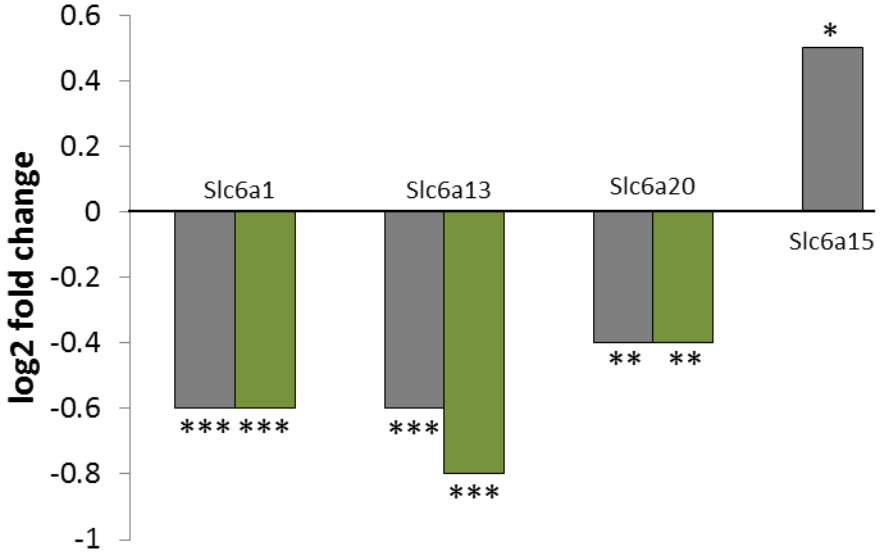


Figure 2.25. mRNA expression of neurotransmitter transporter Slc6a family members in SNNN (green) and SSSN (grey) groups in E21 cortex. Data are presented as log2 fold change from controls (*p≤0.05; **p≤0.01; ***p≤0.001; n=6).

GABA receptors

It was previously demonstrated that GABA receptors are also sensitive to the effects of stress. Therefore we checked GABA receptor expression changes in cortex. Our results showed that *Gabbr2, Gabra5, Gabrb1* were up-regulated in several groups in the offspring of F2 and F3 generations (*Gabbr2* in SNN, SSN, SNNN, and SSSN; *Gabra5* in SNNN and SSSN; *Gabrb1* in

SSSN), whereas *Gabbr1* was down-regulated in cortex of F3 offspring in both the SNNN and SSSN groups.

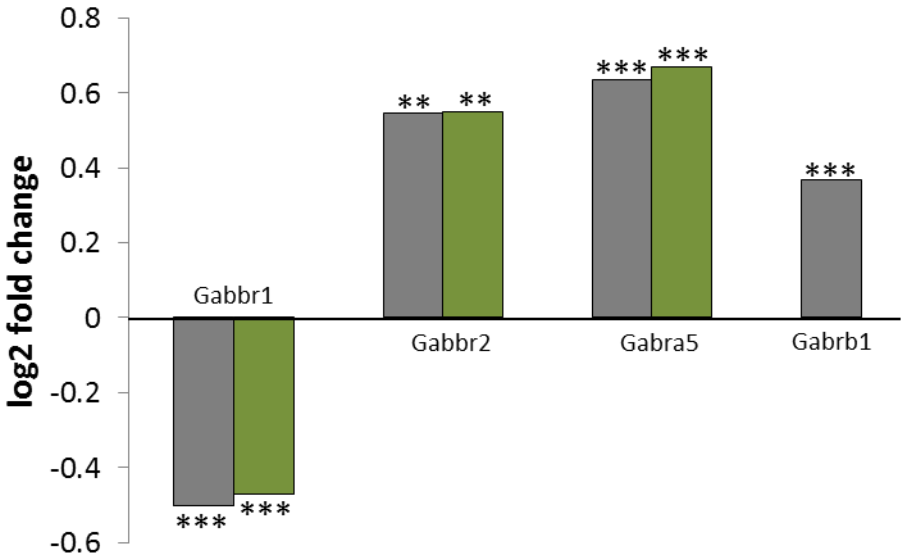


Figure 2.26. GABA receptors expression. mRNA expression in E21 cortex of F3 generation offspring in SNNN (green) and SSSN (grey) groups. Data are presented as log2 fold change from controls (**p≤0.01; ***p≤0.001; n=6).

Glutamate receptors

To verify whether the expression of receptors of another important neurotransmitter, glutamate, was altered, we checked mRNA levels of NMDA, AMPA and kainate receptors. Our results showed that some of the NMDA and kainate receptors subunits were down-regulated in embryonic cortex, including *Grina* (in SNN, SSN and SNNN groups), *Grin1* (in SSN) and kainate receptor subunit Grik5 (in SNN and SSN groups), whereas AMPA receptor subunits *Gria2* and *Gria3* were up-regulated in (SNN, SNNN, SSSN and SNNN, SSSN groups respectively) (Fig. 2.27).

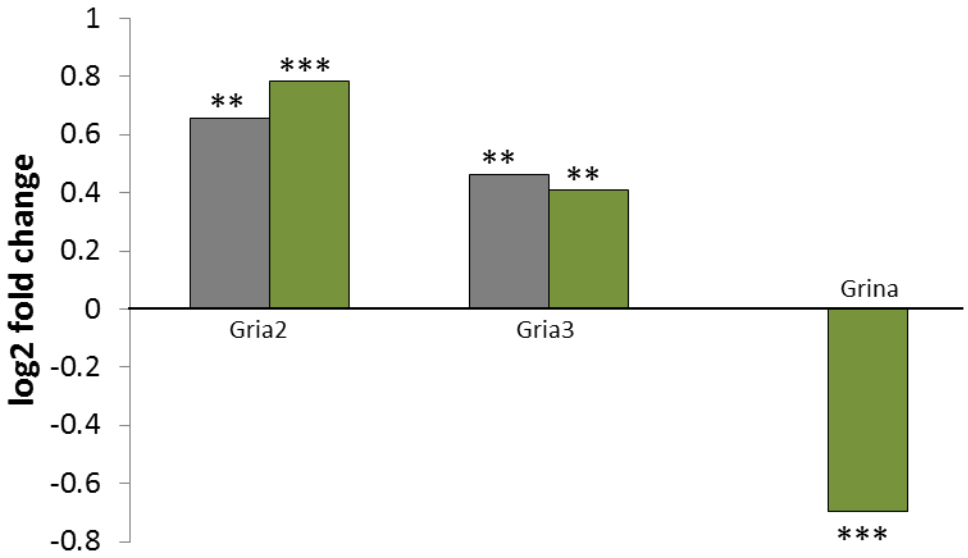


Figure 2.27. Glutamate receptors expression. mRNA expression in E21 cortex of F3 generation offspring in SNNN (green) and SSSN (grey) groups. Data are presented as log2 fold change from controls (**p≤0.01; ***p≤0.001; n=6).

Effects of stress on the expression of genes involved in neurological disorders

Our results indicated that several pathways involved in neurological disorders were activated in embryonic cortex in response to prenatal stress (Table 2.13).

Table 2.13. Number of genes altered in KEGG pathways associated with neurological disorders.

Kegg pathway	F2 generation				F3 generation			
	placenta		cortex		placenta		cortex	
	SNN	SSN	SNN	SSN	SNNN	SSSN	SNNN	SSSN
Glioma	9	X	10	X	14	X	14	12
Huntington's disease	X	X	X	X	X	35	X	29
Amyotrophic lateral sclerosis	X	X	10	X	X	X	13	X
Prion diseases	X	7	X	X	9	10	X	X
Alzheimer's disease	X	22	X	X	X	X	X	X

The glioma pathway was altered in E21 cortex in the F3 generation in both SNNN (14 genes, FE=1.9, p≤0.05) and SSSN (12 genes). In the F2 generation the glioma pathway showed a trend towards activation in the SNN group only (10 genes, FE=1.8, p=0.09). Changes in the expression of genes involved in the glioma pathway were observed in placenta also in the F3 and

F2 generations in non-cumulative lineages only: the SNNN group (14 genes, FE=1.7, p=0.065), and the SNN group (9 genes, FE=2, p=0.085).

Our results showed activation of the amyotrophic lateral sclerosis pathway in the cortex of the offspring in the non-cumulative stress exposure lineage: F3: the SNNN group (13 genes, FE=1.9, p≤0.05), and F2: the SNN group (10 genes, FE=1.9, p=0.075).

Activation of the prion disease pathway was observed in placenta in both F2 and F3 generations. In the F3 generation both stress lineages were affected: SNNN group (9 genes, FE=1.9, p-value=0.095), SSSN group (10 genes, FE=2.1, p≤0.05). In the F2 generation only the cumulative stress exposure group (SSN) was affected (7 genes, FE=2.6, p≤0.05).

Huntington's disease pathway was activated in the cortex of the F3 SSSN group (29 genes, FE=1.4, p=0.07), with similar changes in placenta (35 genes, FE=1.4, p≤0.05).

Activation of the Alzheimer's disease pathway was observed in placenta of F2 generation SSN group only (22 genes, FE=1.4, p=0.09).

Discussion

The effects of stress on fetal and placental development

Successful pregnancy requires proper functioning of the neuroendocrine system that includes not only maternal brain but both fetal brain and placenta (Douglas & Ludwig 2008, Voltolini & Petraglia 2014). Animal and human studies indicated that prenatal stress can affect the activity of the placental glucocorticoid barrier that regulates the metabolism of cortisol (O'Donnell et al 2012) and thus may contribute to the subsequent alterations in the stress response system and glucocorticoid signaling in the brain. Moreover, stress and glucocorticoids activate many biochemical processes in multiple tissues and have a huge impact on cell physiology that consequently induces functional alteration on systemic level (Fowden et al 1998).

Our results demonstrated that prenatal stress activated multiple signalling pathways in the placenta and brain in F2 and F3 generations. Transgenerational changes included disruption of the

placental glucocorticoid barrier, altered expression of imprinted genes and growth factors, neurotransmitter receptors and transporters, altered prostaglandin synthesis, synaptic plasticity, axon guidance, and others. All these biological pathways play important roles in placental function and fetal development and will be discussed in more details in the next chapters. Moreover, we observed alteration of the pathways associated with diseases of the nervous system, such as glioma, amyotrophic lateral sclerosis, prion diseases, Huntington's and Alzheimer's diseases, which also will be discussed below.

Transgenerational effects of stress on placenta function

The placenta plays an important role in fetal growth regulation (reviewed by (Murphy et al 2006)). Some aspects of placental function are critical for human fetal growth and development, including transport of nutrients, such as glucose, amino and fatty acids, production and transfer of growth-regulating hormones. (Murphy et al 2006). All these placental functions can be influenced by prenatal stress during a critical time of embryonic development. Observed changes in placental function in multiple generations in response to prenatal stress will be discussed below.

Prenatal stress disrupts the placental glucocorticoid barrier and *Hsd11b* expression

Prenatal stress involves expression of glucocorticoids from the adrenal gland. Glucocorticoids are steroid hormones that are key mediators of the stress response and exert their function via binding to glucocorticoid and mineralocorticoid receptors (GR and MR) that are expressed in a large variety of tissues. Access of maternal glucocorticoid to the GR in both placenta and fetus is regulated by the glucocorticoid barrier, a major component of which includes 11beta-hydroxysteroid dehydrogenase (HSD11B) enzymes, as well as multidrug resistance P-glycoprotein (ABCB1). HSD11B enzymes (HSD11B1, HSD11B2) catalyze interconversion of active glucocorticoids into their inert metabolites and *vice versa* (Fig. 2.23), whereas ABCB1 enzymes mediate efflux of glucocorticoids from the syncytiotrophoblast (Mark et al 2009). In rodents, two genes, *Abcb1a* and *Abcb1b*, encode ABCB1 (Kalabis et al 2005).

Our results showed significant down-regulation of the major component of placental barrier - *Abcb1b* in the SSN group in the F2 generation only. It was demonstrated in mice that *Abcb1b* mRNA is the predominant placental isoform and that *Abcb1b* mRNA progressively decreased towards the end of the gestation (Kalabis et al 2005). The authors also found a strong correlation between placental *Abcb1b* mRNA and maternal progesterone concentrations, suggesting a potential role of progesterone in regulation of placental *Abcb1b* mRNA (Kalabis et al 2005). Decrease in placental *Abcb1b* mRNA was reflected by the changes in ABCB1 on the protein level over the second half of gestation. The authors suggested therefore that the fetus may become increasingly susceptible to the influences of xenobiotics and natural steroids in the maternal circulation towards the end of the pregnancy (Kalabis et al 2005).

Our results showed that expression of mRNAs encoding HSD11B1, another major component of the placental glucocorticoid barrier, was altered in placenta in different groups after stress exposure. Intriguingly, comparisons of cumulative (SSN) vs. single generation stress exposure (SNN) in F2 generation revealed only two genes being differentially expressed in placenta, including of *Hsd11b1* (that was up-regulated) and of hypothetical protein LOC293871 (down-regulated). *Hsd11b1* converts inactive cortisone to cortisol, the active hormone. Interestingly, similar up-regulation of its expression was observed in F3 generation in both SSSN and SNNN groups. *Hsd11b1* gene expression is associated with increased fetal cortisol concentration at term in humans (Murphy & Clifton 2003) and corticosterone levels in rats (Mark et al 2009). The placental glucocorticoid barrier protects the fetus from maternal glucocorticoids, however, it was demonstrated previously that the expression of *Hsd11b1* mRNA was increased in late gestation, whereas *Hsd11b2* was almost lost (Mark et al 2009). Studies in sheep suggested that the rise of fetal cortisol leads directly to the onset of parturition (Fencl et al 1980, Liggins et al 1973). In humans, fetal corticosterone sulphates were reported to increase suddenly at term (Fencl et al 1980), however the exact role of glucocorticoids in the initiation of parturition in humans remains unclear. Thus, taking into consideration that *Hsd11b1* expression levels increase

in late gestation during normal rat gestation, we can speculate that prenatal stress alters normal functioning of the stress response system, leading to an overexpression of glucocorticoids in rat fetuses at the end of gestation. Higher-than-normal glucocorticoid levels may have, in turn, an adverse effect on the offspring's health later in life. For example, substantial evidence from human and animal studies points towards developmental programming of hypertension, kidney disease and type 2 diabetes mellitus, which are discussed below.

Potential link to chronic renal disease and impaired glucose tolerance

The HSD11B1 enzyme serves to amplify the levels of glucocorticoids locally (Seckl & Walker 2004). Fetal exposure to excess glucocorticoids causes hypertension later in life, which is also a major factor for chronic kidney disease (Chong & Yosypiv 2012). Observations from our lab also indicate that prenatal stress may play a role in the chronic kidney disorder in adulthood (unpublished data). Our results also showed that insulin signaling was altered in both placenta and cortex of F2 and F3 offspring, thus supporting the notion that obesity, type 2 diabetes, glucose intolerance and other metabolic syndromes may be developmentally programmed. Peripheral insulin resistance and pancreatic b-cell dysfunction are two hallmark characteristics of type 2 diabetes mellitus (T2DM). Chronic exposure to psychosocial stressors is a recognized contributing factor that promotes T2DM (Beaudry & Riddell 2012). Taking into consideration that T2DM ultimately results from pancreatic b-cell failure it is possible that prenatal stress has a negative impact on the development of pancreas.

In our study we did not assess the levels of *Hsd11b1* in tissues other than brain and placenta, but it was shown that the enzyme encoded by this gene has an important function in pancreas, liver and adipose tissue. For example, Turban et al. (2012) demonstrated that optimal elevation of pancreatic b-cell *Hsd11b1* is a compensatory mechanism that prevented high-fat diet-induced b-cell failure, suggesting that glucocorticoids are not necessarily suppressive of b-cell function, but rather trigger a U-shaped dose response in the pancreas (Turban et al 2012). In contrast, elevation of *Hsd11b1* in adipose tissue and liver was shown to have a diabetogenic effect

(Morton & Seckl 2008). Studies in both humans and rodents indicate that obese individual have increased activity in adipose tissue (Seckl et al 2004). Mice deficient in HSD11b1 resist both metabolic syndrome and age-related glucocorticoid-associated cognitive impairments (Seckl & Walker 2004).

Hsd11b1 expression is of particular importance during embryonic development. Excessive glucocorticoids and impaired glucocorticoid signaling were shown to have a negative impact on developing pancreas (Gesina et al 2006, Shen et al 2003). It was also demonstrated in a mouse model that transgenic mice with increased glucocorticoid sensitivity in pancreatic b-cells develop diabetes with age (Davani et al 2004).

Thus, summarizing all of the above, prenatal stress and impaired glucocorticoid expression may impair normal development of different organs at the later fetal stages. Altered function of the pancreas during embryonic development in response to prenatal stress may therefore program predisposition to metabolic syndrome later in life.

Interestingly, inhibition of *Hsd11b1* is now believed to have great therapeutic potential in AD, major depression, type 2 diabetes and other disorders (reviewed in (Vitku et al 2014)). For example, Sandeep et al. (2004) showed that administration of a 11 beta-hydroxysteroid dehydrogenase inhibitor, carbenoxolone improved verbal fluency in healthy elderly men and verbal memory in patients with type 2 diabetes (Sandeep et al 2004). The authors demonstrated that *Hsd11b1* but not *Hsd11b2* mRNA is expressed in human hippocampus, frontal cortex and cerebellum, thus highlighting its role in adult brain function (Sandeep et al 2004). Beneficial effects of HSD11b1 inhibition were also demonstrated in aging animals (Sooy et al 2010). Partial deficiency or short-term inhibition of 11 HSD11b1 improved cognitive function in aging mice. Thus, it would be interesting to verify whether HSD11b1 inhibition during development would have a similar effect on aging.

Transgenerational effects of stress on glucocorticoid receptors signaling and prostaglandin synthesis

Glucocorticoids play multiple functions including essential role in normal fetal development where they promote maturation of various fetal tissues and prepare embryo for extrauterine life (Figueroa et al 2005, Fowden et al 1998, Sutherland et al 2012). Moreover, glucocorticoids were shown to regulate prostaglandin synthesis in placenta (Patel et al 1999). HSD11B1 that was discussed above is also involved in the prostaglandin synthesis and regulation pathway (Salomonis et al 2005). Prostaglandins are bioactive hormone-like lipids that act in autocrine and paracrine fashion to regulate multiple biological processes, but also are required for normal function of female reproductive system (Fortier et al 2008). Prostaglandins are mediators of pain, fever, inflammation, hypertension (Fortier et al 2008) and play a role in contraction of the smooth muscle of the uterus (Ruan et al 2011) and cervical ripening (Witter 2000). Prostaglandin pathway gene expression in human placenta was shown to be affected by preterm labour (Phillips et al 2014).

Our results showed that expression of several genes involved in prostaglandin synthesis and regulation pathway were altered in F2 and F3 generations in both stress lineages (Fig.2.23, Table 2.9). Significantly altered genes include endothelin receptor B (*Ednrb*), platelet-derived growth factor A chain (*Pdgfa*), histone acetyltransferase (*Kat5*), prostaglandin-endoperoxidase synthase 2 (*Cox2*), several annexins (*Anxa1,2,5*, and *7*), two prostaglandin synthases (*Ptgis, Ptges2*), prostaglandin F2 receptor inhibitor (*Ptgfrn*), prostaglandin dehydrogenase 1 (*Hpgd*), prostaglandin transporter (*Slco2a1*), cholesterol side-chain cleavage enzyme (*Cyp11a1*), progesterone receptor membrane component 1 (*Pgrmc1*), hydroxysteroid (11-beta) dehydrogenase 1 (*Hsd11b1*) and several inflammation markers (*Gata6, Cdx-2, Il11ra1, Il6ra, Adam17*). Most of these genes had altered expression in the same direction in all groups in placenta of F2 and F3 generations. Some of the genes from this pathway were also altered in E21 cortex. *Ednrb* was significantly up-regulated in SSN, but not in SNN group in placenta in F2

generation, suggesting that stress alters prostaglandin synthesis in placenta via its action on endothelin receptors.

Transgenerational control of embryonic and neonatal growth by imprinted genes and growth factors

Evidence from both human and animal studies showed that prenatal stress may lead to low birth weight and altered HPA axis activity later in life (Andrews & Matthews 2004). Glucocorticoids in particular have been shown to inhibit fetal growth in both animals and humans (Fowden 1995, Seckl 1994). We did not assess the weight of the pups at E21, but the results of separate experiments with the same stress paradigm showed that prenatal stress caused significant elevation in the weight of pups at postnatal day 1 (P1) in both cumulative and single generation stress exposure lineages.

Our observation of increased weight of the pups at P1 in response to prenatal stress in F2, F3 generations might be the result of the altered function of placental insulin-like growth factor (IGF) axis and changes in the expression of imprinted genes. IGF axis is a complex system that includes insulin-like growth factors (IGF1, IGF2), their receptors (IGF1R, IGF2R), IGF-binding proteins (IGFBP-1 to IGFBP-6) and associated IGFBP degrading enzymes. Some of the components of the IGF axis are also imprinted. We will discuss the changes in the expression of the IGF axis components and imprinted genes below.

Genomic imprinting refers to the epigenetic marking of the parental origin of certain chromosomal domains that takes place at a small subset of genes termed imprinted. Imprinted genes therefore display a parent-of-origin allele-specific gene expression in somatic tissues (Herman & Feil 2008). To date, more than 100 imprinted genes have been identified in mice, and over 50 in humans (Wilkins 2014). The most significantly represented function among imprinted genes in mammals is regulation of embryonic growth and development (Barlow & Bartolomei 2014), however there is an increasing evidence that imprinted genes influence brain function and behaviour in adult life by affecting neurodevelopmental processes (Cheverud et al 2008, Lorenc et

al 2014, Wilkinson et al 2007). Many of imprinted genes are expressed in placenta and the developing embryo where they mainly function as growth promoters if they are paternally expressed (i.e., *Igf2, Peg1,Peg3, Rasgrf1, Dlk1*) or growth repressors in case of maternal imprinting (i.e., *Igf2r, Gnas, Cdkn1c, H19, Grb10*) (Barlow & Bartolomei 2014). Another important category of imprinted genes includes those that play an important role in behavioural and neurological defects (e.g., *Nesp, Ube3a, Kcnq1*) (Barlow & Bartolomei 2014). Interestingly, despite the fact that most of the studies on genomic imprinting have focused on the effects of embryonic development, some evidence suggests that genomic imprinting can also influence behaviour that is controlled by the adult brain, such as maternal care or social dominance (Cheverud et al 2008, Curley 2011, Garfield et al 2011, Wilkins & Haig 2003, Wolf et al 2008, Wolf & Hager 2009). For example, a study by Cheverud et al. (2008) demonstrated that genomic imprinting affected normal variation in adult body composition phenotype in mice (Cheverud et al 2008). Intriguingly, a recent study by Lorenc et al. (2014) suggested that mate choice decisions in mice appear to be influenced by paternally inherited cues (Lorenc et al 2014). Thus, the above mentioned studies highlight that importance of genomic imprinting exceeds embryonic development.

Our results showed that out of more than 150 imprinted genes that are known to-date, expression of 20 genes was altered in different groups in both placenta and embryonic cortex in F2 and F3 generations (see Tables 2.10, 2.11, Fig. 2.24).

In addition, our results showed that expression of several imprinted genes, including *Plagl1*, *Cdkn1c* and *Igf2* in placenta and *Cdkn1c* in cortex was common for F2 and F3 generations for both lineages. Interestingly, overexpression of *Plagl1* was implicated in transient neonatal diabetes mellitus type 1 in humans (Mackay & Temple 2010), whereas *Cdkn1c* and *Igf2* were shown to be of crucial importance for placental efficiency in rodents (Angiolini et al 2006).

Interestingly, Drake et al. (2011) using a three generation rat model of programming by initial prenatal glucocorticoid overexposure showed generation-specific effects on the expression

of imprinted genes in fetus and placenta in F1 and F2 (Drake et al 2011). The authors treated pregnant dams with dexamethasone (synthetic corticosteroid that crosses placenta) from G15-20. It was demonstrated previously that this stress model reduced birth weight and induced hypertension, insulin resistance and anxiety-related behaviour in the offspring (Nyirenda et al 1998, Welberg & Seckl 2001). Results of Drake et al. (2011) showed that prenatal dexamethasone treatment induced reduction in the placental and fetal weight at E20, as well as birth weight of the offspring in the F1 generation. Intriguingly, generation-specific parent of origin effects were observed in the F2 generation. Similarly to the F1 generation, reduction of weight of placenta and fetal body at E20 was observed in F2 progeny of fathers that were prenatally exposed to dexamethasone treatment. Interestingly, prenatal dexamethasone treatment had the opposite effect on the F2 progeny if the mothers, but not the fathers were exposed. Maternal dexamethasone treatment caused increase in placental and fetal weight at E20. However, birth weight was decreased in all F2 offspring, regardless of the paternal or maternal dexamethasone exposure lineage (Drake et al 2011). Drake et al. (2011) also assessed the levels of mRNA expression of candidate imprinted genes (including *Igf2, Cdkn1c, Phlda2, H19, and Slc22a3*) in fetal liver and placental tissues during late gestation. Expression of several genes that were altered in the experiment of Drake et al (2011) was also altered in our experiment, despite the differences in experimental design. This may indicate that prenatal stress may have some general effects on the expression of imprinted genes.

Interestingly, prenatal exposure to endocrine disrupters was also shown to alter expression of imprinted genes in both placenta and embryo (Kang et al 2011, Susiarjo et al 2013). For example, Susiarjo et al. (2013) observed the disruption of expression of several imprinted genes including *Snrpn, Igf2, Cdkn1c,* and *Ascl2* in E9.5 and E12.5 embryos and placentas in mice after maternal bisphenol A (BPA) exposure during late stages of oocyte development and early stages of embryonic development.

Importantly, clusters of imprinted genes are known to be regulated by imprinting control regions (ICRs) that are characterized by DNA methylation of one allele, thus DNA methyltransferases (DNMTs) might be implicated in the observed changes in the expression of imprinted genes. Our results showed that *Dnmt1* was down-regulated in SNNN group (FC=0.7, $p \leq 0.001$) in cortex, whereas *Dnmt3a* was up-regulated in F2 generation SNN group (FC=1.9, $p \leq 0.05$), but not SSN. *Dnmt3a* was also up-regulated in F2 generation in placenta in both SNN (FC=1.4, $p \leq 0.05$) and SSN groups (FC=1.4, $p \leq 0.001$), but not in F3 generation. Interestingly, increased *Dnmt3a* mRNA level in placenta in response to prenatal stress was also shown in the experiment by Pena et al (2012). The authors also observed an increase in *Dnmt1* mRNA expression in E20 fetal cortex in response to chronic restraint stress during gestational days 14-20 (Jensen Pena et al 2012)

mTOR signaling in growth control and disease

The mammalian target of the rapamycin (mTOR) signalling pathway is another important player in the regulation of growth and homeostasis (Laplante & Sabatini 2012). The mTOR signaling pathway integrates both internal and a variety of external environmental signals and is a central regulator of cell growth, metabolism, proliferation and survival (Laplante & Sabatini 2009). This pathway was shown to be activated in different pathological conditions, including cancer (Guertin & Sabatini 2007), obesity (Yang et al 2012), insulin resistance type 2 diabetes (Blagosklonny 2013) and neurodegeneration (Bove et al 2011). Therefore, it attracted a broad clinical interest in mTOR inhibitors (Laplante & Sabatini 2009). mTOR is a serine/threonine protein kinase that can be inhibited by rapamycin that is an approved immunosuppressant drug used to prevent the rejection of transplanted organs and as a treatment for cardiovascular diseases (to block restenosis after angioplasty). Interestingly, a growing body of evidence showed that rapamycin has a neuroprotective effect in several animal models of neurodegenerative disorders, including Alzheimer's, Parkinson's, Huntington's diseases and spinocerebellar ataxia type3 (reviewed in (Bove et al 2011)).

The activity of the mTOR signaling pathway was shown to be decreased in human placentas with intrauterine growth restriction and increased in obese women giving birth to large babies, possibly via its role in the regulation of amino acid transporters in the placenta (Jansson et al 2013, Roos et al 2007, Roos et al 2009). Interestingly, our results showed that mTOR signaling was altered in placenta in the F3 generation only, suggesting a generation-specific transgenerational effect of stress on this pathway.

Transgenerational effects of stress on insulin signalling

Insulin is the primary hormone that regulates the maintenance of blood glucose levels in the organism via stimulation of the uptake of glucose by liver, muscle, and adipose tissue. Insulin resistance plays a central role in the pathogenesis of several metabolic disorders, including obesity, type 2 diabetes, glucose intolerance, and cardiovascular disorders. The pathophysiology of insulin resistance involves a complex network of signaling pathways activated by insulin receptor in fat, liver and muscle, however insulin action in tissues not classically considered insulin sensitive, such as brain or placenta, may also play an important role in glucose homeostasis (Boileau et al 2001, Saltiel & Kahn 2001). Insulin receptors are present in placenta, however the role of insulin signaling in placenta is not clear (Boileau et al 2001). Our results showed activation of the insulin signaling pathway in both placenta (29 genes altered in the pathway) and cortex (22 genes) in SNNN group in the F3 generation. This pathway was also activated in placenta in F2 generation in both SNN (17 genes) and SSN (19 genes) groups (see Table 2.8).

The canonical insulin signaling pathway activation starts with the insulin binding to its receptors, whereas alternative pathway activation also involves flotillin in this process and likely functions in the fine-tuning of the canonical pathway (Banning et al 2014). Our results showed no changes in the expression of insulin receptor mRNA (*Insr*), whereas flotillin1 (*Flot1*) was down-regulated in SNNN placenta (FC=0.6, $p \leq 0.0001$) and in SNNN cortex (FC=0.7, $p \leq 0.001$). Flotillins are highly conserved proteins that were originally discovered to be up-regulated in

neurons during axonal regeneration after lesioning of the optic nerve (Schulte et al 1997). Flotillins have been implicated in a myriad of processes that include endocytosis, signal transduction and regulation of the cortical cytoskeleton, yet understanding of the molecular mechanism and their multiple functions remains a challenge (Otto & Nichols 2011). Besides, insulin signaling, flotillins were shown to play a role in several other signaling pathways, including EGFR, FGFR, and neurotrophin receptor signaling (reviewed in (Banning et al 2014)). Interestingly, it has been demonstrated recently that flotillins have a key role in endocytosis of glutamate and dopamine transporters in mammals (Cremona et al 2011), some of which were altered in our experiment and will be discussed later. Flotillins were also recently found to be expressed in the human placenta (Walton et al 2013).

As can be seen on Fig S1 (Supplemental materials) the activation of insulin signaling pathway also depends on the *Ptprf* expression. Protein tyrosine phosphatase receptor type F (*Ptprf*) also known as leukocyte antigen related (*Lar*) is a receptor type IIA protein that has been implicated in neural development, cancer and diabetes (Chagnon et al 2004). *Ptprf* is an inhibitor of insulin signaling and an increased expression level of this protein was found in the insulin-responsive tissue of obese, insulin-resistant individuals, and may contribute to the pathogenesis of insulin resistance (Ahmad et al 1997, Ahmad et al 1995). *Ptprf* overexpression was shown to induce insulin resistance in transgenic mice (Zabolotny et al 2001), whereas reduction in *Ptprf* expression resulted in an increase of insulin receptor tyrosine phosphorylation and kinase activity in the rat hepatoma cell line (Kulas et al 1995). Moreover, *Ptprf* gene variability was found to be associated with insulin resistance, obesity and coronary artery disease in type 2 diabetes (Menzaghi et al 2008, Miscio et al 2004). Our results showed down-regulation of *Ptprf* mRNA in SNNN placenta (FC=0.8, $p \leq 0.001$) and in SNNN cortex (FC=0.8, $p \leq 0.001$).

Altered insulin signaling in placenta may also play a role in pregnancies with complications. For example, endogenous expression levels of insulin signaling components were

shown to be altered in human term placental tissues from woman with gestational diabetes mellitus in comparison to normal controls and maternal obesity (Colomiere et al 2009).

Role of insulin and insulin signaling in the CNS

Insulin signaling in the central nervous system (CNS) emerges as an exciting field of research (Banks et al 2012). Insulin signaling plays a vital role in the developing and adult brain (Duarte et al 2012, McNay & Recknagel 2011, Plum et al 2005 (Duarte et al 2012, McNay & Recknagel 2011). Growing body of scientific evidence highlighted an important role of insulin in cognitive processes and brain control of food intake (Gerozissis et al 2001, Zhao et al 2004, Zhao et al 1999). Low brain insulin levels and/or signaling were shown to be associated with impaired learning and memory, and age-related neurodegenerative diseases (Talbot et al 2012). Insulin receptor transduction in the central nervous system ultimately results in the diverse biological effects of insulin signaling, including inhibition of apoptosis, tau phosphorylation, regulation of amyloid precursor protein secretion and gene transcription (e.g. of hypothalamic neuropeptides) (Plum et al 2005). Administration of insulin directly to the brain has an obesity-preventive effect, which results in the loss of appetite with the subsequent reduction of the body weight (Air et al 2002, Carvalheira et al 2003, McGowan et al 1993, Woods et al 1979). Whereas inhibition of insulin signaling in the brain was shown to have an appetite-stimulating effect, leading to the body weight gain and peripheral insulin resistance (Bruning et al 2000, McGowan et al 1990, Obici et al 2002).

CNS insulin resistance is associated with Alzheimer's disease (Talbot et al 2012), depression (Rasgon & Kenna 2005, Wroolie et al 2014), and impaired baroreceptor gain in pregnancy (Azar & Brooks 2011, Daubert et al 2007). Interestingly, intranasal insulin administration was shown to modulate verbal memory and plasma amyloid beta peptide in a dose-dependent manner in adult human with memory impairments (Reger et al 2008), thus highlighting its therapeutic potential.

Stress and insulin signaling

Direct relationships between prenatal stress exposure and later changes in metabolic systems in the offspring are not well studied. One epidemiological study by Entringer et al. (2008) showed that prenatal stress exposure is associated with insulin resistance in young adults (Entringer et al 2008). The authors demonstrated that healthy young adults whose mothers experienced major psychosocial stress during pregnancy (including relationship conflicts, death or severe illness of someone close, severe financial problems, etc.) had significantly higher insulin responses in oral glucose tolerance test in comparison to those, who were not prenatally stressed (Entringer et al 2008).

Transgenerational effects of stress on the brain development

Our results showed that prenatal stress exposure of F0 mothers led to the alteration in several gene expression pathways that are of particular importance for the brain development, such as axon guidance, long-term potentiation and long-term depression that play a role in learning and memory, and tight junctions that are crucial for the integrity of the blood-brain barrier. These pathways will be discussed in more details below.

Transgenerational effects of stress on axon guidance

Our results of KEGG pathways analysis showed that expression of 26 genes was altered in the axon guidance pathway in both SNNN and SSSN groups. This pathway was also activated in the F2 generation in SNN (19 genes) and SSN (17 genes) groups. Activation of the pathways that participate in axonal growth, trafficking of synaptic vesicles and neurotransmitter release was also shown to occur in the hippocampus of 23-day-old offspring of prenatally stressed rats (Bogoch et al 2007), thus indicating that changes in synaptic function that occur during prenatal period may have a long-lasting effect. Moreover, Bogoch et al. (2007) showed that hippocampal gene expression changes in response to prenatal stress were accompanied by anxiety-like behavior (Bogoch et al 2007). Repeated restraint stress and corticosterone injections during pregnancy

were also shown to impair axon sprouting and reorganization in hippocampus and prefrontal cortex of rat pups at postnatal days P7-14 (Jutapakdeegul et al 2010).

Transgenerational effects of stress on synaptic plasticity in the cortex

Our results of KEGG pathway analysis showed that the long-term potentiation pathway was altered in the cortex of F3 generation offspring in both SNNN (15 genes) and SSSN (18 genes) groups. Expression of 13 genes was altered in long–term depression (LTD) pathways. Long-term potentiation (LTP) and LTD are two forms of synaptic plasticity that are thought to play a key role in memory formation. Yang et al. (2006) reported previously that prenatal stress impaired LTP, but facilitated LTD in the hippocampus of prenatally stressed rat offspring at the age of 5 weeks (Yang et al 2006). Moreover, Yang et al. (2006) showed that prenatal stress enhanced the effects of acute stress on the hippocampal synaptic plasticity and impaired spatial learning and memory in young rat offspring (Yang et al 2006). Our findings indicate the existence of possible transgenerational programming of brain functions and support the notion that prenatal stress alters synaptic plasticity in the developing brain that possibly leads to memory and learning deficits in the offspring.

Transgenerational effects of stress on development of blood-brain barrier tight junctions

The blood-brain barrier (BBB) is an interface between the peripheral circulation and the CNS that is formed by cerebral microvascular endothelial cells connected by tight junctions (TJs) that regulate the passage of ions and molecules through the paracellular pathway. TJs are the structural equivalent of the BBB and can change the degree of permeability of the barrier in response to external stimuli, physiological and pathological conditions (Gonzalez-Mariscal et al 2008). During early development intraneural vessels originate by angiogenesis starting from day 11 in rat and give rise to the extended vascular system, but until E17 developing BBB appears to be non-functional (Kniesel et al 1996). The external BBB of pial vessels in rat embryos has a low transendothelial electrical resistance up to E20 and thus it is considered immature (Wolburg & Lippoldt 2002). Disruption of the tight junctions of the BBB is a hallmark of many

neuropathologies, including stroke, Alzheimer's diseases and multiple sclerosis (Huber et al 2001). Our results showed that expression of 25 genes that belong to the tight junctions' pathway was altered in E21 cortex in F3 generation offspring (SNNN) in response to prenatal stress of parental generation (F0). Similarly, expression of 25 genes in this pathway was altered in SSSN group. However, in F2 generation offspring tight junction pathway was activated in SNN (19 genes), but not in SSN group.

Liebner et al. (2008) showed that Wnt/β-catenin signaling controls the development of the BBB in the developing mouse brain (Liebner et al 2008). Our results showed that Wnt signaling pathway (19 genes) was altered in embryonic cortex in SNN group in F2, but not F3 generation. β -catenin is also a part of tight junction pathway and our results showed down-regulation of β-catenin mRNA (*Cttnnb1*) in cortex in F3 generations in both SNNN and SSSN groups. Interestingly, it was recently demonstrated that β-catenin mediates stress resilience through Dicer1/microRNA regulation (Dias et al 2014). β-catenin was also implicated in neuropsychiatric illnesses, including depression and schizophrenia (Beaulieu et al 2004, Brennand et al 2011, Gould et al 2007, Madsen et al 2003). Dias et al. (2014) demonstrated that overexpression of β-catenin in nucleus accumbens (NAc) prevented depression-like behaviour in mouse models, whereas blocking β-catenin signaling in NAc promoted susceptibility to stress in mice subjected to a sub-threshold defeat procedure (Dias et al 2014). Thus, observed down-regulation of *Cttnnb1* in E21 cortex of F3 generation offspring, whose great-grandmothers were stressed during pregnancy, may be indicative of higher susceptibility to stress in these animals.

The role of stress on the expression of neurotransmitter receptors and their transporters

Neurotransmitters are endogenous chemicals that are packaged into synaptic vesicles and transmit signals across a synapse from one neuron to another. They exert their function through binding to the specific membrane receptors that could be ligand-gated ion channels, like $GABA_A$, AMPA, NMDA and serotonin receptors, or G protein-coupled receptors, like $GABA_B$, and dopamine receptors. The majority of neurotransmitters are known for their role in neuronal

communication in the mature brain, however more and more evidence indicates an important role that neurotransmitter receptors play in the developing brain (Lujan et al 2005). For example, glutamate and GABA are abundantly expressed during embryonic development (Benitez-Diaz et al 2003, Miranda-Contreras et al 1998). Some evidence indicates that ionotropic glutamate receptors modulate pre- and postnatal neurogenesis (Di Giorgi Gerevini et al 2004), moreover, they are also expressed in neuronal progenitors and neural stem cells (Pachernegg et al 2013). Thus, each step of CNS development involves appropriate expression and function of neurotransmitters and their receptors (Lujan et al 2005).

It was demonstrated previously that prenatal stress induces long-lasting changes in neurotransmitter receptors in the brain (Berger et al 2002, Henry et al 1995). Our results showed transgenerational changes in mRNA expression of several neurostransmitter receptors, including dopamine, serotonin, GABA, and glutamate receptors in embryonic cortex in response to prenatal stress in parental generation. Our results demonstrated that dopamine receptor 2 (*Drd2*) mRNA was down-regulated in SNN, SSN in F2 generation, and in SNNN group only in F3 generation. Whereas mRNA of one of the receptors of another neurotransmitter, serotonin, (*Htr7*) was up-regulated in F3 both SNNN and SSSN.

Both acute and chronic stresses are known to have a negative impact on the functioning of the dopaminergic system (reviewed in (Pani et al 2000)). Prenatal stress was shown to increase the *Dr2* binding in the nucleus accumbens (Berger et al 2002, Henry et al 1995), frontal cortex, hippocampus and other brain regions (Berger et al 2002). The role of D2 receptor density was also demonstrated in thermoregulation (Kiyatkin et al 1991) and restraint stress (Tomic & Joksimovic 1991). A recent study by Hill et al. (2014) addressed the possible role of alteration in BDNF and dopamine receptors expression in response to stress in relation to future risks of developing schizophrenia (Hill et al 2014). Using a rat model, the authors tested the 'two hit' hypothesis which states that two major disruptions during development may be involved in the pathophysiology of schizophrenia. Their results showed that the combination of neonatal maternal

separation and young-adult corticosterone treatment increased the mRNA expression of two dopamine receptors (*Dr2* and *Dr3*) in medial prefrontal cortex and nucleus accumbens (Hill et al 2014). Another recent study that utilized a mouse model of social stress showed that *Drd2* was 1.8 fold change down-regulated in amygdala (Azzinnari et al 2014). Thus, these studies indicate that the directionality of changes in dopamine receptors expression in response to stress can be different in various brain regions.

Glutamatergic synaptic transmission was also shown to undergo significant changes during development (Ben-Ari et al 1997). Glutamate is another important neurotransmitter that is required for normal neurodevelopment. Glutamate promotes neuronal migration, differentiation and plasticity (Komuro & Rakic 1993). Glutamate is the primary excitatory neurotransmitter in the brain that binds to three ionotropic receptors: 1) α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor, 2) N-methyl-D-aspartate (NMDA) receptor and 3) kainaite receptor. Our results showed that some of these receptors were altered in embryonic cortex of F2 and F3 offspring (Fig. 2.28).

NMDA receptor is a glutamate receptor that plays a pivotal role in synaptic plasticity and memory function (Li & Tsien 2009). Blockade of the NMDA receptor was shown to impair synaptic plasticity and compromised learning and memory in rodents. It was demonstrated that blocking NMDA receptors for only a few hours during late fetal life triggered apoptotic neurodegeneration in the developing rat brain (Ikonomidou et al 1999). During first 2 weeks of neonatal life NMDA receptors are primary mediators of glutamatergic fast excitatory neurotransmission in the rat brain (Ben-Ari et al 1997).

Prenatal stress was shown previously to have long-term effects and influence the expression of glutamate receptors in the adult brain (Berger et al 2002, Fumagalli et al 2009). These effects were shown to be prevented by environmental manipulations during early postnatal period, such as cross-fostering experiments (Barros et al 2004).

Our results showed that mRNA expression of several neurotransmitter transporters was down-regulated by prenatal stress in the embryonic cortex in the offspring of F2 and F3 generations. Neurotransmitter transporters that were altered by stress included two GABA transporters *Slc6a1* and *Slc6a13*, taurine and beta-alanine transporter *Slc6a6*, glycine transporters *Slc6a9* and *Slc6a20* (Fig. 2.26). *Slc6a15* is a transporter of neutral amino acids and it was up-regulated in SSSN group in F3 generation offspring. Interestingly, *Slc6a15* was implicated in major depression (MD) (Kohli et al 2011). Decreased *Slc6a15* expression might alter neuronal circuits related to susceptibility for MD (Kohli et al 2011).

Interestingly, our results showed that expression of *Slc6a9* and *Slc6a13* was also decreased in placenta in SNN and SSS groups respectively. Amino acid transporters play important role in nutrient transport across the placenta (Murphy et al 2006). Alterations in amino acid transport by placenta and uptake by the fetus were demonstrated in fetal growth restriction (Jansson et al 1998, Norberg et al 1998). Taurine is an essential amino acid for the fetus that executes various physiological functions important for fetal growth and central nervous system development (Norberg et al 1998). It was demonstrated that sodium-dependent transport of taurine was reduced in IUGR placentas compared with those from normal pregnancies (Norberg et al 1998).

Transgenerational effects on neurotransmission: SNARE interactions in synaptic vesicles

The SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) protein superfamily is important element of the intracellular trafficking machinery (Ungar 2003 SNARE protein structure and function). The SNAREs are central players in all trafficking steps of the secretory pathway, including neurotransmission (Palfreyman & Jorgensen 2008). Intracellular vesicular trafficking and membrane fusion is crucial for the nervous system development and proper functioning of the neural circuits. Evidence from mouse models showed that synaptosomal-associated protein 25 kDa (SNAP-25) which is part of SNARE complex, plays an important role in action potential dependent release of several neurotransmitters during development (Tafoya et al 2006). SNAP-25 was shown to play crucial role in evoked

glutamatergic and cholinergic transmission in central neurons and at neuromuscular junctions (Washbourne et al 2002), in calcium-triggered catecholamine release from adrenal chromaffin cells (Sorensen et al 2003) as well as it was shown to be critical for the evoked GABA release during development (Tafoya et al 2006). It was also recently demonstrated that SNARE-mediated exocytosis is a prerequisite for axon branching in murine cortical neurons (Winkle et al 2014).

Our results showed that expression of two genes that encode predominant neural SNARE complex proteins *Snap25* and *Vamp2* (vesicle-associated membrane protein 2) were up-regulated in E21 cortex in the offspring of F3 generation in SSSN group. *Snap25* was also up-regulated in SNNN group in F3 generation and both cumulative and non-cumulative stress exposure line in F2 generation.

Cumulative vs. single generation stress exposure in the F3 generation

Our comparison of altered KEGG pathways in cumulative vs single generation stress exposure groups in F3 generations showed that most of the altered pathways were common between two stress lineages. Interestingly, two important metabolic pathways, citrate cycle and oxidative phosphorylation were activated in the SSSN group but not SNNN.

The placenta requires constant and sufficient amounts of energy to perform its protective and endocrine functions, as well as to provide the fetus with nutrients, vitamins and oxygen, and to eliminate waste. Glucose that is transported across the placenta from the maternal circulation meets placental and fetal energy needs via glycolysis and the citric acid cycle (Bax & Bloxam 1997, Illsley 2000). Activation of energy production pathways in the SSSN group may reflect an increase in energy demand in the placenta in response to prenatal stress and may lead to an increase in oxidative stress, possibly contributing to the disease states. It was reported, for example, that increased amounts of mitochondria in placentas with preeclampsia contribute to oxidative stress via generation of excessive superoxide (Wang & Walsh 1998).

Similarly to placenta, our results showed that a few pathways were differently activated in the E21 cortex in our SSSN group, including: progesterone-mediated oocyte maturation, Fc

gamma R-mediated phagocytosis, fatty acid metabolism, other glycan degradation, several pathways in cancer, Huntington's disease, adherens junctions, GnRH signaling pathway, and long-term depression.

Adherens junctions, GnRH signaling and long-term depression pathways may be of particular importance for brain development. Both tight and adherens junctions are part of the blood-brain-barrier (BBB), disruption of which is a hallmark of many CNS pathologies, including stroke, Alzheimer's disease, multiple sclerosis and bacterial meningitis (Huber et al 2001). β-catenin, which is an important member of adherens junction pathway, was down-regulated in the SSSN group in E21 cortex. Interestingly, it was reported recently that β-catenin mediates stress resilience through Dicer1/microRNA regulation (Dias et al 2014). Gonadotropin-releasing hormones (GnRHs) stimulate the production of the β-subunit of human chorionic gonadotropin and may also play a role in trophoblast invasion (Sasaki & Norwitz 2011). Long-term depression (LTD) is an activity-dependent reduction in the efficacy of neuronal synapses lasting hours or longer following a long patterned stimulus. LTD together with long-term potentiation (LTP) are widely accepted models for the cellular and molecular mechanisms that underlie synaptic changes during learning and memory (Massey & Bashir 2007). Interestingly, it was shown that ability to express LTD is developmentally dependent and is down-regulated in rat hippocampus (Kemp et al 2000, Wagner & Alger 1995). Thus, our findings support the notion that prenatal stress causes changes in learning and memory system, possibly via alteration in LTD.

F2 generation vs F3 generation

Some of the pathways were similarly altered in placenta and cortex of the offspring in both F2 and F3 generations, including long-term potentiation, neurotrophin signaling, MAPK signaling, Gap junction, etc. (Table 2.8). In general**,** expression of more genes from each pathway was altered in F3 generation in comparison to F2 (Table 2.8). Some changes, however, were generation-specific. For example, in cortex, Wnt signaling pathway was altered only in the offspring of F2 generation in SNN group, whereas activation of SNARE interactions pathway was

unique for F3 generation SNNN group. Similarly, in placenta, mTOR signaling pathway was activated in SNNN and SSSN groups in offspring of F3, but not the F2 generation, whereas activation of vascular smooth muscle contraction, TGF-beta and GnRH signaling pathways was unique for the SNN group of the F2 generation. These changes could reflect a generation-specific effects of stress, or alternatively, this could be a result of the analysis cut-off, where some changes did not reach statistical significance and therefore were not represented in the results.

Since changes in the F3 generation may reflect transgenerational programming, it is important to trace changes down from F3 back to F2 generation. Interestingly, most of the activated pathways from F3 SNNN group were also altered in F2 SNN, except for mTOR signaling pathway in placenta and ECM-receptor interaction and SNARE interactions pathways in cortex. The role of SNARE interactions in synaptic vesicles and mTOR signaling was discussed in previous chapter. TGF-beta signaling and ECM receptor interaction will be discussed below.

TGF-beta signaling

The transforming growth factor beta (TGF-beta) is an important regulator of placental development and functions (Jones et al 2006, Xuan et al 2007). TGF-beta is considered to be the most potent autocrine negative regulator of proliferation in epithelial tissues (Lehnert & Akhurst 1988, Schmierer & Hill 2007). Alterations in TGF-beta signaling are implicated in serious human diseases including cancer, fibrosis, wound-healing disorders, familial primary pulmonary hypertension and hereditary hemorrhagic telangiectasia (Blobe et al 2000, Massague et al 2000). Decreases in TGF-beta signaling molecules with gestational age was observed in normal human placentas, and significant down-regulation of TGF-beta signaling was noticed in choriocarcinoma (Xuan et al 2007). All of the genes except of *Erk* in the pathway were up-regulated in the SNN group in the F2 generation. Knockout models and embryo culture experiments implicate TGF-beta pathway members, such as activins, TGFbs, nodal and bone morphogenetic proteins (BMPs) in promoting pre- and post-implantation embryo development (Jones et al 2006).

ECM-receptor interaction

The extracellular matrix (ECM) signaling regulates cell behavior and orchestrates multiple cell functions during tissue formation (Lukashev & Werb 1998). The proper functioning of ECM is important for neurological development and its disruption can lead to synaptic and neuronal loss (reviewed in (Bonneh-Barkay & Wiley 2009)). ECM molecules, their receptors at the cell surface and cell adhesion molecules are implicated in several diseases of the CNS, including Alzheimer's and Parkinson's diseases, epilepsy, schizophrenia, addiction, and multiple sclerosis (Berezin et al 2014).

The structure of brain ECM differs from systemic one and includes the perineuronal net (PNN). PNN is a neuronal cell surface structure composed of a variety of proteoglycans. It is important to note that gene that encodes one of the most important enzymes involved in cleavage of ECM proteoglycans *Adam17* was significantly down-regulated in SNNN group (FC=0.76, $p \leq 0.001$). Thus, decrease in *Adam17* expression in the cortex may indicate an increase in ECM receptors signaling activity. Expression of genes that encode several ECM molecules, that were identified as crucial modulators of the formation, maturation and plasticity of synapses (Bonneh-Barkay & Wiley 2009) was found to be altered in SNNN group, including laminin (*Lamb2*, FC=0.7, $p<0.0001$) and reelin (*Reln*, FC=1.4, $p \leq 0.01$). Thus, modulation of ECM functioning in the brain can affect learning and memory via interference with induction or maintenance of LTP that was demonstrated in transgenic mouse models (Brakebusch et al 2002, Ethell & Ethell 2007, Evers et al 2002). More and more evidence indicates that ECM may be involved in the process of neurodegeneration (Baig et al 2005, Bruckner et al 1999, Morawski et al 2012). Drugs targeting major ECM receptors have a potential for treatment of stroke, multiple sclerosis and AD.

While mature ECM and PNN may play a role in the repair of CNS, interaction between cells and the ECM in developing brains is important for the cell migration and axon guidance (Berezin et al 2014, Kwok et al 2011). Our results also showed the activation of pathways involved in axon guidance (discussed above). Moreover, components of ECM were shown to play an important

role in other cell functions and pathways that we found to be altered, including maintenance of blood-brain barrier (Baeten & Akassoglou 2011), glutamate neurotransmitter receptors (AMPA, GABA, kainate) activation, expression and mobility (Chen et al 2008, Frischknecht et al 2009, Groc et al 2007), long-term potentiation (Wright et al 2002).

Stress alters pathways associated with brain diseases

The concept of fetal origins of adult disease or fetal programming was developed by David Barker and colleagues based on the observation that a low birth weight increases the risks of cardiovascular disease and type 2 diabetes in later life (Barker et al., 1993). This concept proposes that fetal adaptations to the intrauterine and maternal environments shape the structure and function of organs, leading to permanent physiological alterations in adulthood (Swanson et al., 2009). Therefore, this approach suggests that the predisposition to diseases in adulthood is "programmed" in utero. Barker's early observation about the role of the fetal environment in the future development of cardiovascular disease has since been extended to other diseases, including conditions affecting the brain, such as psychiatric disorders.

Interestingly, our results of the pathway analysis in placenta and embryonic cortex indicated that several pathways associated with diseases of the brain were altered, such as glioma, amyotrophic lateral sclerosis (ALS), prion disease, Huntington's and Alzheimer's diseases. It is worth noting that most of these diseases are associated with the oxidative stress response in the CNS.

Conclusions

Epidemiological and animal evidence indicates that the prenatal environment permanently programs later physiological functioning of the organism and affects the risks of metabolic, cardiovascular, neuroendocrine and mental health disorders in adulthood. A concept of fetal origins of adult disease or fetal programming was first introduced by David Barker and colleagues, based on the observation that low birth weight increases the risks of cardiovascular diseases and type 2 diabetes later in life (Barker et al 1993a). Since then, major scientific evidence

suggests that a possible link between future adverse health effects and early-life embryonic experience lies in altered glucocorticoid signaling in response to excessive exposure to this major stress hormone during development. Human epidemiological data shows clear signs of early-life developmental programing where low birth weight or rapid postnatal weight gain are independent risk factors of overweight and obesity later in life as well as higher risks of diabetes and metabolic syndrome. Abnormal postnatal weight gain is often the consequence of an altered intrauterine environment which can be caused by high psychosocial stress levels perceived by the mother during pregnancy. Some human epidemiological data shows a direct relationship between stress during pregnancy and altered insulin responses in the adult offspring. More intriguingly, a few epidemiological and animal studies provide a link between ancestral exposure to adverse environment and health outcomes in the subsequent generations, suggesting that fetal programming of adult diseases may also have transgenerational consequences. More research is needed to verify the molecular mechanisms of transgenerational programming, which can change our current perception of prenatal stress and highlight a key importance of implementing new stress-preventative strategies to eliminate stress-programmed health risks in future generations. Despite a surging interest in fetal programming, molecular mechanisms of it remain largely unknown. Using our experimental model we attempted to shed light on the extent of transgenerational programming by prenatal stress. Our experiments allowed us to answer how which molecular pathways may be involved in transgenerational programming. We found transgenerational changes in gene expression in the placenta and embryonic cortex. Our results showed transgenerational effects of prenatal stress on the expression of imprinted genes and growth factors, the functioning of the glucocorticoid barrier in placenta, etc. Many biological pathways were altered in offspring of the F2 and F3 generations in response to stress in the parental generation, including those involved in brain development, synaptic plasticity, neurotransmission, and cell signaling in the embryonic cortex. Surprisingly, our results showed no effect of prenatal stress on F1 offspring. Given that we observed major changes in multiple

biological pathways in F2 and F3 generations offspring that were not exposed to prenatal stress, we can speculate that the effects of prenatal programming are not detectable until later during development. We assessed gene expression on E21 in both placenta and embryonic cortex. Observed changes in embryonic cortex in F2 and F3, but not in F1 may indicate that germlines were more susceptible to the effects of prenatal stress than the developing embryonic brain. Moreover, it could be that changes in the brain of F1 offspring were not detectable until later in life. Similarly, the absence of altered gene expression in the placenta of the parental generation, but its occurrence in subsequent generations may again indicate that F1 offspring were indeed affected by prenatal stress, but these differences became more prominent and measurable only later, when prenatally stressed females were pregnant themselves. Possibly, prenatal stress caused very subtle changes in brain functioning and signaling processes in E21 cortex, but these changes resulted in altered stress response of the organism later in life that was reflected by altered gene expression in placenta during pregnancy. Alternatively, observed changes in gene expression in F2 and F3 can represent the consequence of the effect that prenatal stress had on the germline or developing fetal organs in the F1 offspring that were not assessed (e.g. pancreas). For example, it is possible that prenatal stress of the parental generation altered the development of the pancreas in the F1 fetus that led to the alteration of the insulin signaling in the adult brain of F1offspring and was transmitted to subsequent generations. It is also possible that prenatal stress exposure caused permanent epigenetic changes in placenta and/or brain in F1. The role of the miRNA expression and DNA methylation in placenta and embryonic brain in response to prenatal stress in multiple generations will be discussed in the next chapters.

Chapter 3: Transgenerational effects of gestational stress on miRNA expression

Introduction

About a third of the world's population is afflicted by cognitive and mental health disorders such as depression, which are commonly related to stress. Early life stress in particular has been recognized as a key factor in determining the vulnerability to neurodevelopmental disorders, cognitive impairments and mental illness later in life (Talge et al 2007a). For example, children exposed *in utero* to maternal psychological stress, caused by trauma, bereavement or natural disasters, are at a higher risk of schizophrenia (Huttunen & Niskanen 1978, Khashan et al 2008, Khashan et al 2011b, Kinney et al 1999a, Myhrman et al 1996a, Selten et al 1999b, van Os & Selten 1998a), autism (Beversdorf et al 2005), depression (O'Connor et al 2002a, O'Connor et al 2003), bipolar disorder (Perroud et al., 2014), and impaired language development (Brouwers et al., 2001; O'Connor et al., 2002; King and Laplante, 2005). Although most studies on prenatal stress focused on the hypothalamic-pituitary-adrenal (HPA) axis and the hippocampus (Meaney et al 2007, Oberlander et al 2008), recent studies have also demonstrated morphological and epigenetic changes in the prefrontal cortex (PFC) that may explain these behavioural impairments (Michelsen et al 2007, Muhammad et al 2012, Yao et al 2014, Zucchi et al 2013). In spite of this considerable evidence linking early adverse experiences and later mental health, little is still known about the mechanisms leading to the experience-dependent programming of lifetime health.

Experimental studies have provided insights into experience-dependent regulation of gene expression and neuronal plasticity, suggesting that epigenetic mechanisms are central to the rapid adaptation to stressful environmental conditions (Feng et al 2007, Francis et al 1999, Weaver et al 2005). Most studies have focused on DNA methylation, in particular experience-related methylation of the glucocorticoid receptor gene (*Nr3c1*) (McGowan et al 2009a, Oberlander et al 2008, Weaver et al 2005). However, striking evidence has suggested a central role of small non-coding RNAs, in particular microRNAs (miRNAs), in the regulation of genes essential to

neuronal development and function (Krichevsky et al 2003, Schratt et al 2006) (reviewed here (Sun et al 2013)). For example, the developing human brain displays distinct temporal and spatial expression patterns of miRNAs (Moreau et al 2013). Notably, our previous findings showed that miRNA regulation of gene expression in the rat brain is highly responsive to stress (Babenko et al 2012a) and particularly affected in the frontal cortex of stressed rat mothers with corresponding changes in their offspring (Zucchi et al 2013). Although miRNAs have now been recognized as biomarkers of diseases of the brain, including schizophrenia, autism, anxiety, depression and bipolar disorder (reviewed in (Babenko et al 2012b), (Miller & Wahlestedt 2010)), their causal role in perinatal programming of these disorders is not clear.

Small non-coding RNAs, including miRNAs, have been detected in mammalian sperm (Johnson et al 2011, Krawetz 2005), suggesting that they may potentially contribute to the transmission of epigenetic memory to subsequent generations. Short interfering RNA-mediated epigenetic changes were demonstrated to be inherited across at least three generations in *Caenorhabditis elegans* (Gu et al 2012). The role for miRNAs in transgenerational epigenetic inheritance was supported by bioinformatic analyses (Sharma, 2014). A recent study in mice showed that stress alters miRNA expression patterns, and the respective miRNAs injected into unexposed oocytes were transmitted to F2 progeny along with the relevant behavioural and metabolic responses (Gapp et al., 2014). Furthermore, we showed previously that stress generates miRNA signatures related to disease in peripheral tissues, which are passed on to subsequent generations (Yao et al 2014). These findings indicate that miRNAs represent key factors in generating a transgenerational epigenetic memory. To prove transgenerational inheritance of experience-driven epigenetic signatures one would expect to find the signatures of stress memory in the unexposed female F3 generation (Skinner, 2008). Thus, the aim of the present study was to identify heritable miRNA signatures possibly linked to mental health using a new model of non-cumulative and cumulative generational maternal stress.

Materials and methods

Animals

For the description see Animals section in Chapter 2.

Experimental groups

For the experimental design see Experimental groups section in Chapter 2.

Time course

For the time course description see Time course in Chapter 2.

Stress paradigm

Stress paradigm is described in Chapter 2.

Brain tissue dissection and RNA extraction

For the details on tissue dissection and RNA extraction see Chapter 2.

miRNA profiling

miRNA profiling was performed using Illumina GAIIx sequencing platform (Illumina, San Diego, CA, U.S.A.). Small non-coding RNA libraries were generated from 42 samples (SN, SNN, SSN, NNN, SSSN, SNNN, NNNN groups) of total RNA in three biological replicates in each group using the TruSeq small RNA library construction kit according to the manufacturer's protocol (Illumina, San Diego, CA, U.S.A.). Briefly, the 3′ and 5′ adapters were ligated to small RNAs from the total RNA sample followed by reverse transcription PCR amplification. PCR was performed with two primers that anneal to the ends of adapters and contain indexes. Subsequently, the libraries with unique indexes were pooled together. Then the cDNA was gel-purified using a TBE PAGE gel and then concentrated by ethanol. Following a successful library quality control by qPCR, flow cell cluster generation was performed using a cBot. Single end multiplexed sequencing was done using the Illumina GAIIx platform with the total of 36 cycles.

Base calling and demultiplexing of sequencing reads generated by the Illumina GAIIx platform was performed using the CASAVA v 1.8.1. software pipeline with default settings. After demultiplexing, adapter trimming was performed using Cutadapt software

(http://code.google.com/p/cutadapt/) with options specified to search for adapters anywhere in the read sequence and retain only sequences in 17-27 nucleotide, quality trimming was performed with Sanger quality score cutoff of 30. Quality of the sequencing libraries was assessed after adapter trimming using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Data analysis and statistical analysis

Normalization and statistical tests were performed using DESeq bioconductor package as described in the user's manual (Anders and Huber 2010). Reads with the sum raw counts ≤ 5 across all libraries that participated in a particular comparison were excluded from the analysis. Precise mapping of all assorted small RNAs was done using MicroRazerS v 1.0 software with default settings (i.e. the first 16 nt-long ones were matched, no mismatch allowed) (Emde et al 2010). MiRNAs with <0.1 False Discovery Rate were considered statistically significant.

Western immunoblotting

For protein isolation, 30-50 mg of tissue was lysed and sonicated in 0.2-0.4 mL of 1% sodium dodecyl sulfate (SDS). The lysates were cleared using centrifugation. The protein content was determined using the Bradford protein determination assay (BioRad, Hercules, CA). Equal amounts of lysate protein were subsequently separated on 6–15 % SDS-polyacrylamide gels and transferred to PVDF membranes (Amersham Hybond™-P, GE Healthcare) at 4° C for 1.5 hours. Western immunoblotting was conducted using the well-established protocol (Tryndyak et al 2006). The membranes were incubated with polyclonal/monoclonal antibodies against mTOR, IGF1-Rβ, TGF-β (Cell Signaling Technologies) or BDNF, DNMT3a, GR, anti-beta Actin (Santa Cruz Biotechnology) or FGFR3 (Abcam Inc., Cambridge, MA) at 4° C overnight. Antibody binding was revealed through the incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and the ECL Plus immunoblotting detection system (GE Healthcare, Piscataway, NJ). Chemiluminescence was detected using BioMax MR films (Eastman Kodak, New Haven, CT). Signals were quantified using NIH ImageJ 1.63 software and

normalized to loading controls (non-stressed animals from NNN group). The results are presented as mean ± standard deviation. The statistical analyses were conducted using one-way ANOVA, with Bonferroni Post Hoc comparisons. P-values less than 0.05 were considered significant.

Results

miRNA profiling of E21 cortex and placenta in three generations (female offspring of F1, F2, F3 generations)

miRNA expression analysis of the thee generations (F1, F2, F3) of female pups was performed using a Illumina GAIIx next generation sequencing platform. E21 cortex samples of three animals per group (n=3) from each of the following groups were used for the analysis: SN, SS, SNN, SSN, NNN, SNNN, SSSN, NNNN. Placentas attached to the embryos were also used for the profiling. Our results showed that there were no significant differences in miRNA expression in the cortex between control and stressed animals in the F1 and F2 generations. The F3 generation revealed two miRNAs differentially expressed in cumulative-stress-exposure line in comparison to controls (SSSN vs. NNNN): miR-409-5p was up-regulated (fold change (FC) =16.4, $p<0.0001$), whereas miR-331 was down-regulated (FC=0.3, $p<0.0001$). In the F0-stress-exposure line four miRNAs were differentially expressed in the cortex of G21 pups in comparison to control animals (SNNN vs. NNNN): miR-331 was down-regulated (FC=0.4, $p<0.0001$), whereas other miRNAs were up-regulated: miR-409-5p (FC=15.7, $p<0.0001$), miR-99b (FC=3.2, $p=0.001$), miR-100 (FC=2.2, $p=0.001$). No changes in miRNA expression in the cortex of control animals (NNNN vs. NNN) were found (Table 3.1).

Table 3.1. MiRNA expression data in E21 cortex in three generations F1, F2, F3. MiRNAs with <0.1 false discovery rate were considered statistically significant and are represented in red.

Generation	Comparison	# of miRNAs changed	miRNAs
F1	**SN vs. NNN**	0	n/a
F2	**SNN vs. NNN**	0	n/a

	SSN vs. NNN	0	n/a
	SSN vs. SNN	0	n/a
F3	**SNNN vs. NNNN**	4	rno-miR-331
			rno-miR-409-5p
			rno-miR-100
			rno-miR-99b
	SSSN vs. NNNN	2	rno-miR-409-5p
			rno-miR-331
	SNNN vs. SSSN	0	n/a
F2 vs. F3	**NNN vs. NNNN**	0	n/a

miRNA expression analysis of placenta showed no significant differences between stressed and control animals in the F1 generation (SN vs. NNN) and in the F0-stress-exposure line in comparison with controls in the F2 generation (SNN vs. NNN), however, six miRNAs were differentially expressed in the F2 cumulative-stress-exposure line (SSN vs. NNN) in placenta. Two out of six miRNAs were up-regulated: miR-409-5p (FC=6.3, $p<0.0001$) and miR-493* (FC=3.1, $p<0.0001$), whereas four miRNAs were down-regulated in SSN vs. NNN: miR-9 (FC=0.3, $p<0.0001$), miR-3596d (FC=0.5, $p<0.001$), miR-331 (FC=0.3, $p<0.001$), and miR-29a (FC=0.3, $p<0.0001$). Results of the miRNA expression in placenta in the F3 generation revealed significant changes in both cumulative- and F0-stress exposure lines. Seven miRNAs were altered in SSSN vs. NNNN comparison, including two miRNAs that were significantly down-regulated (miR-331 (FC=0.2, $p<0.0001$), miR-154 (FC=0.4, $p<0.0001$)). By contrast, expression of five miRNAs was increased (miR-99b (FC=2.3, $p<0.0001$), miR-322* (FC=2.4, $p<0.0001$), miR-30e* (FC=2.2, $p<0.0001$), miR-409-5p (FC=12, $p<0.0001$), and miR-871* (FC=2.4, $p<0.0001$)). Eleven miRNAs were altered in the SNNN vs. NNNN comparison in placenta. Three miRNA were down-regulated, including miR-331 (FC=0.2, $p<0.0001$), miR-675* (FC=0.4, $p<0.0001$), and miR-370 (FC=0.5, $p=0.002$). By contrast, eight miRNAs were up-regulated, including miR-871* (FC=2.3, $p<0.0001$), miR-322* (FC=2.3, $p<0.0001$), miR-409-5p (FC=11.2, $p<0.0001$), miR-100 (FC=2, $p<0.0001$), miR-501* (FC=3.7, $p=0.002$), miR-153 (FC=3.7, $p=0.002$), miR-

181a (FC=2.1, p=0.003), and miR-99b (FC=2.5, p<0.0001). INNNN vs. NNN comparison between controls showed differential expression of three miRNAs, including miR-871* (FC=0.5, p<0.0001), miR-134 (FC=2.0, p=0.001), and miR-154 (FC=3.0, p=0.001) (see Table 3.2).

Table 3.2. MiRNA expression data in placenta in three generations F1, F2, F3. MiRNAs with <0.1 false discovery rate were considered statistically significant and are represented in red.

Generation	Comparison	# of miRNAs changed	miRNAs
F1	**SN vs. NNN**	0	n/a
F2	**SNN vs. NNN**	0	n/a
	SSN vs. NNN	6	rno-miR-409-5p
			rno-miR-9
			rno-miR-29a
			rno-miR-493*
			rno-miR-3596d
			rno-miR-331
	SSN vs. SNN	0	n/a
F3	**SNNN vs. NNNN**	11	rno-miR-331
			rno-miR-409-5p
			rno-miR-100
			rno-miR-871*
			rno-miR-99b
			rno-miR-322*
			rno-miR-181a
			rno-miR-675*
			rno-miR-153
			rno-miR-370
			rno-miR-501*
	SSSN vs. NNNN	7	rno-miR-409-5p
			rno-miR-30e*
			rno-miR-871*
			rno-miR-322*
			rno-miR-331
			rno-miR-154
			rno-miR-99b
	SNNN vs. SSSN	0	n/a
F2 vs. F3	**NNN vs. NNNN**	3	rno-miR-871*
			rno-miR-134
			rno-miR-154

Some of the altered miRNAs were common for both F2 and F3 generations, whereas some miRNA were unique for different groups (Fig. 3.1).

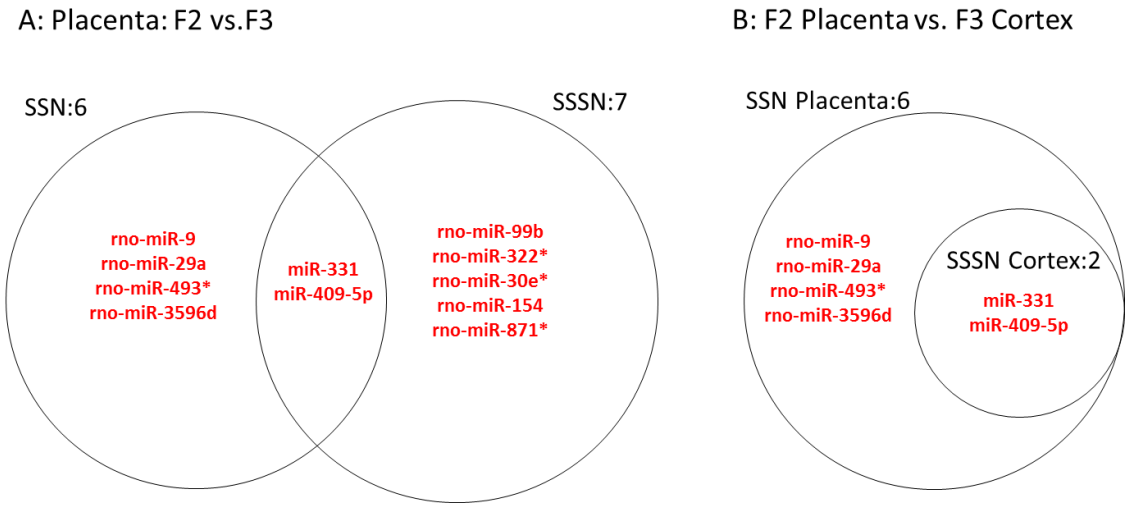


Figure 3.1. MiRNA expression changes in the cumulative stress exposure groups (F2 vs. F3)**.** The Venn diagrams illustrate genes expressed in common and uniquely in each group. Numbers in brackets indicate sample size.

All altered miRNAs in E21 cortex of the offspring from F3 generation were also altered in placenta (Fig. 3.2).

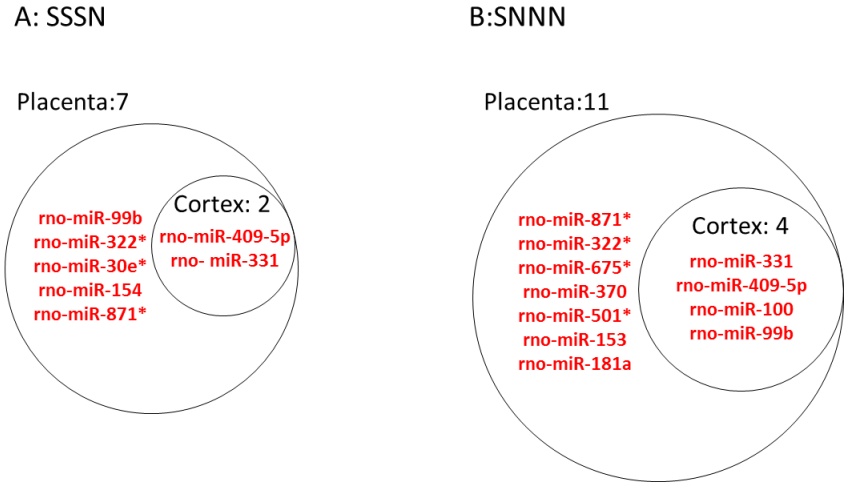


Figure 3.2. MiRNA expression comparison in cortex vs. placenta in the F3 generation. A: SSSN vs. NNNN, B: SNNN vs NNNN. Numbers indicate the total number of miRNAs changed.

Some of the miRNAs differentially expressed in the F3 generation were unique for the cumulative- or F0-stress exposure lines, whereas some of the miRNAs were common for all comparisons (see Fig. 3.3). MiR-100 and miR-99b were chosen for further analysis of the targets since they were uniquely altered in SNNN cortex.

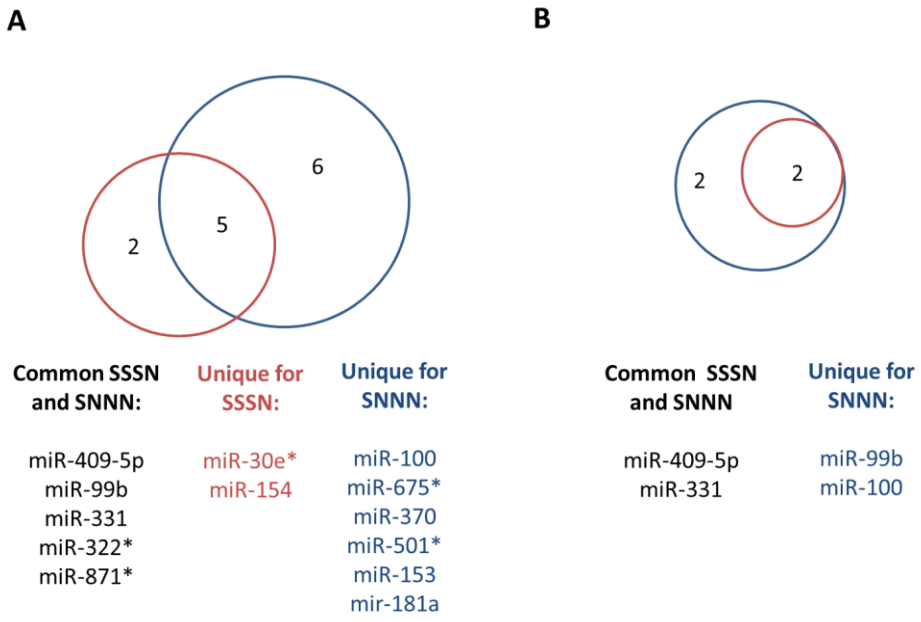


Figure 3.3. Cumulative- vs. F0-generation stress exposure effects in miRNA expression in placenta (A) and E21 cortex (B) in the F3 generation. Numbers indicate the total number of miRNAs changed.

Overall, miRNA expression analysis showed significant changes in expression of 17 miRNAs among different groups in different generations. To verify whether changes in those miRNAs were gradual in each generation or similar in different groups and in different tissues we plotted fold changes of each miRNA expression across three generations in both cortex and placenta (Fig. 3.4). For more details on the miRNA expression data of each miRNA see Table S4. Seven out of 17 miRNAs are biologically conserved (broadly conserved among most vertebrata) (Table 3.3).

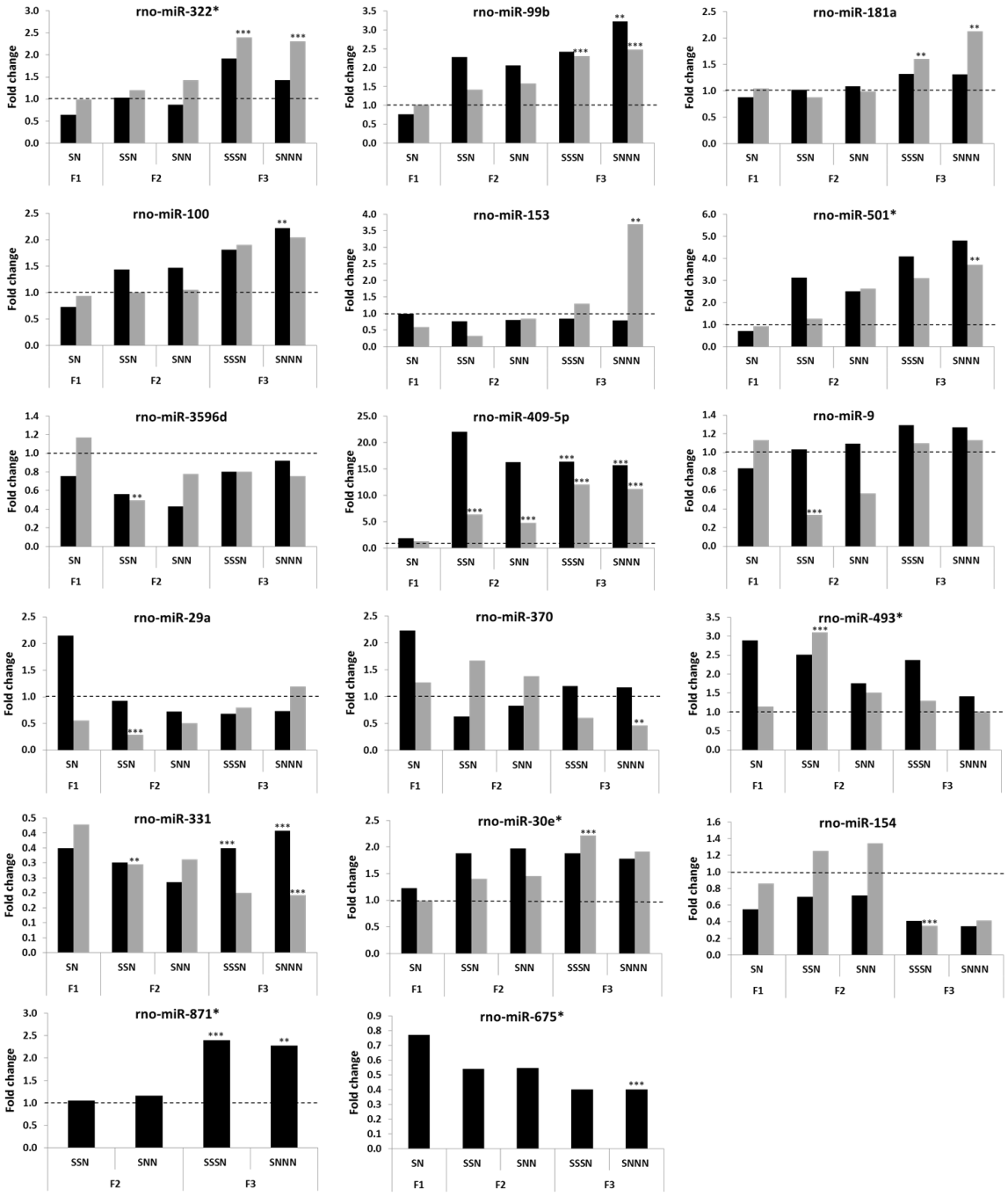


Figure 3.4. MiRNA expression comparisons in cortex (black) and placenta (grey) of three generations of offspring. Data are represented as fold change (FC) from controls (n=3).

Table 3.3. Biological conservation of miRNAs. Broadly conserved: conserved across most vertebrates (usually to zebrafish); conserved: conserved across most mammals (usually not beyond placental mammals); poorly conserved: all others (Friedman et al 2009).

evolutionary conservation	miRNA
broadly conserved miRNA	miR-100
	miR-153
	miR-181a
	miR-99b
	miR-9
	miR-29a
	miR-30e*
conserved miRNA	miR-154
	miR-370
poorly conserved miRNA	miR-871*
	miR-675*
	miR-501*
	miR-409-5p
	miR-493*
	miR-3596d
	miR-322*
	miR-331

To verify whether cumulative stress exposure causes similar changes in the miRNA expression patterns in cortex of offspring across three generations we plotted fold changes of miRNA expressions in each of the following groups together: SN, SSN, SSSN. The results demonstrated that six miRNAs had a trend towards increase in expression in embryonic cortex in cumulative stress exposure line across three generations, including miR-9, miR-322, miR-100, miR-99b, and miR-501*, whereas expression of miR-29a, and miR-493 tended to decrease across generations in cumulative stress exposure line (Fig. 3.5). No obvious linear patterns in expression of other miRNAs were observed across generations. For changes in miRNA expression in the cumulative stress exposure line across three generations in placenta see Fig. 3.6.

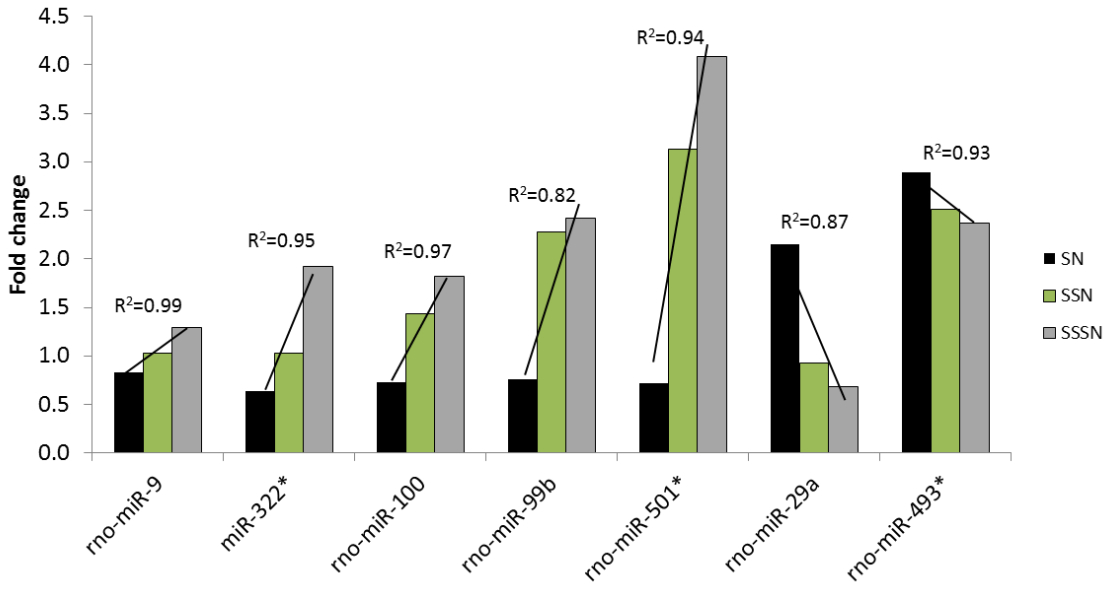


Figure 3.5. Summary of data highlighting the trend towards an increase in expression of miR-9, -322*, -100, -99b, 501* and decrease in the expression of miR-29a and -493* with each generation in E21 cortex of the offspring from the cumulative stress exposure lineage.

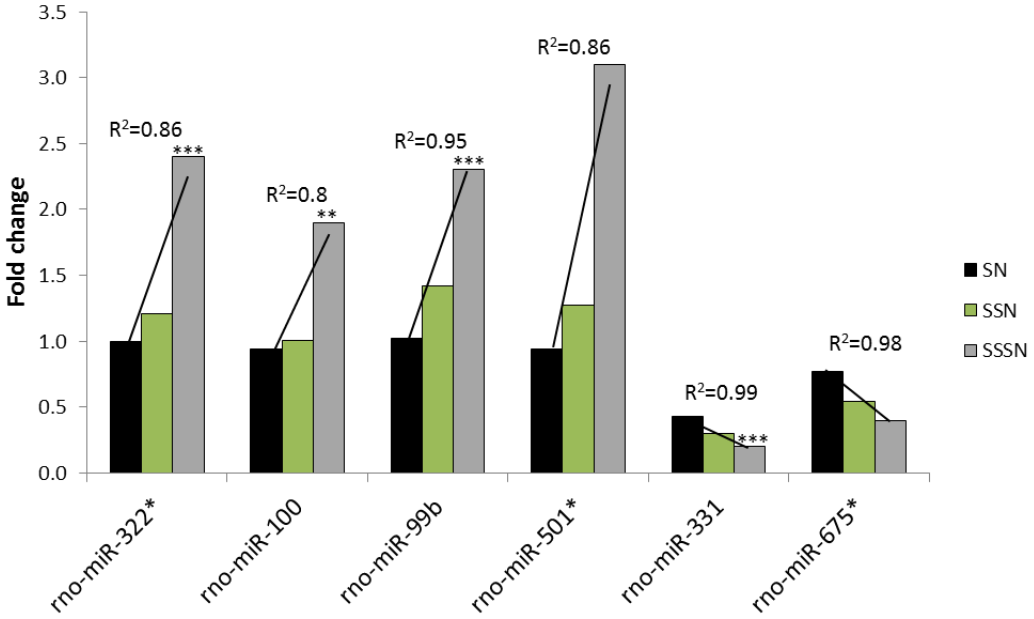


Figure 3.6. Trend towards an increase in expression of miR-322*, -100, -99b, 501*and decrease in expression of miR-331, and -675 with each generation in placenta in the cumulative stress exposure lineage.

Similar comparisons across three generations of placental miRNA expression revealed some trends in non-cumulative stress exposure line (SN vs. SNN vs. SNNN groups). A decrease

in expression across three generations was observed for miR-331, and miR-675*, whereas expression of miR-322*, miR-100, miR-99b, miR-501*, miR-153, and miR-409-5p tended towards an increase (Fig. 3.7). MiRNA expression changes in the single-generation stress exposure line in the embryonic cortex of the offspring across three generations are summarized in Fig. 3.8.

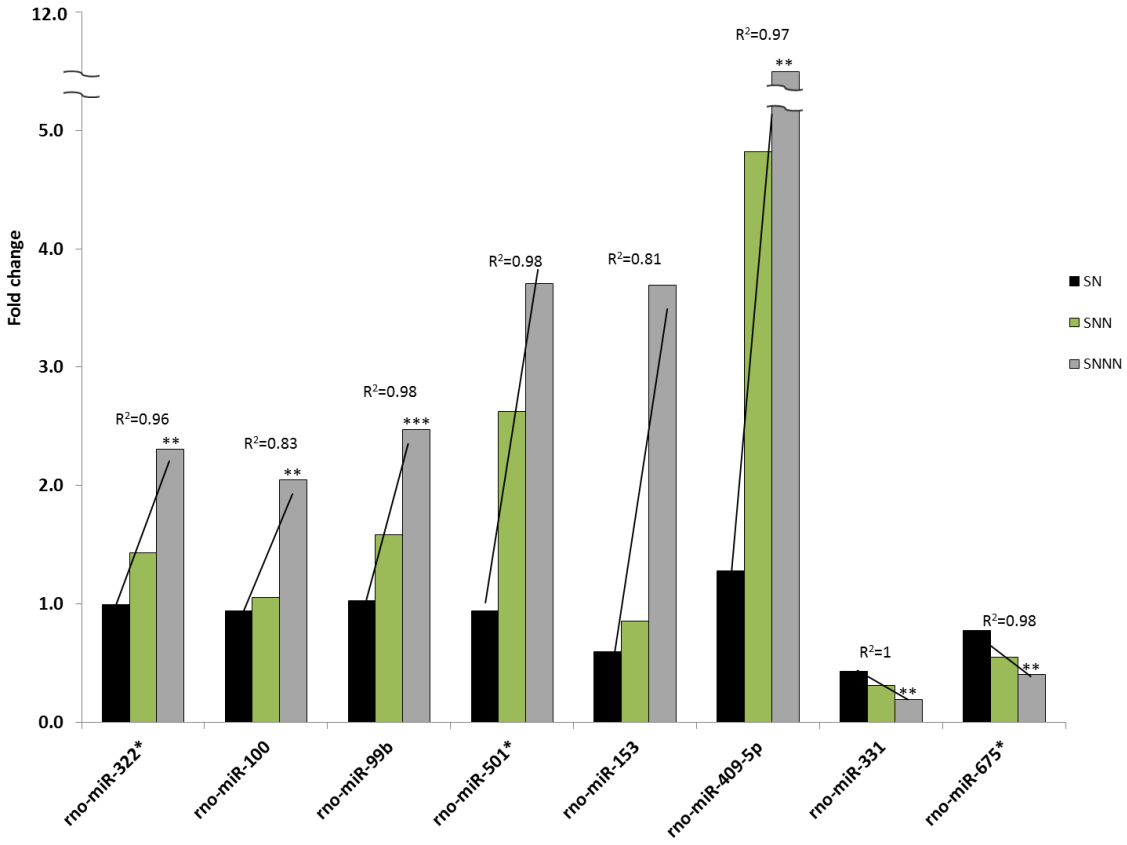


Figure 3.7. Trends towards an increase in expression of miR-322*, -100, -99b, -501*, -153, -409-5p or decrease in expression of miR-331 and -675* with each generation in placenta in single-generation stress exposure lineage.

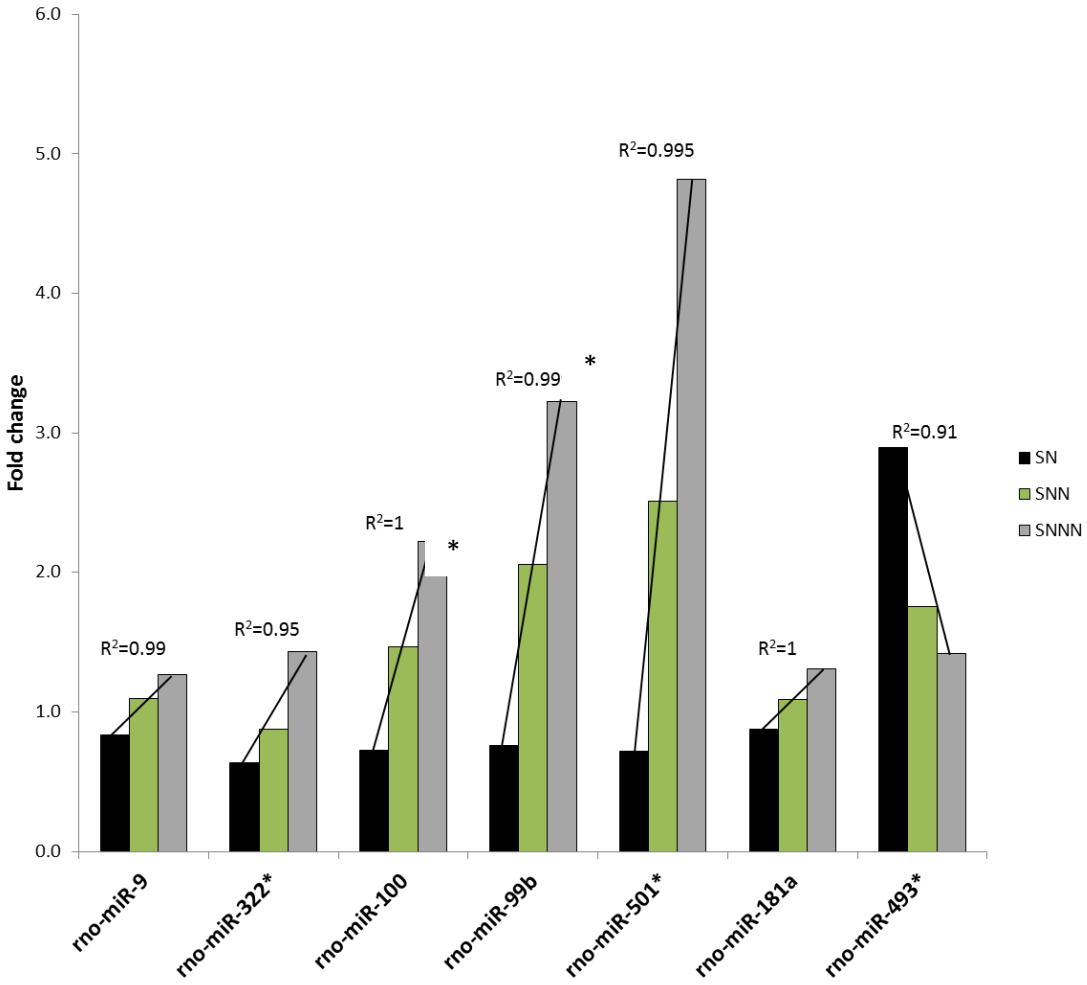


Figure 3.8. Trends towards an increase in expression of miR-9, -322*, -100, -99b, -501*, -181a, and decrease in expression of miR-493* with each generation in single-generation stress exposure line in E21 cortex.

miRNA profiling of P133 prefrontal cortex in F3 generation (adult females)

To verify whether miRNA expression patterns in the brain of E21 female pups of F3 generation offspring were persistent to adulthood, we analyzed miRNA expression in the prefrontal cortex of adult F3 females on postnatal day 133 (P133). Our results showed that one miRNA was altered in the F0-stress-exposure group in comparison to controls (SNNN vs. NNNN) – miR-92b-3p (FC=1.4, $p<0.0001$). Nine miRNAs were altered in the SSSN vs. NNNN group, three being up-regulated: miR-92b-3p (FC=1.4, $p<0.0001$), miR-92a-3p (FC=1.7, $p<0.0001$), and miR-128-3p (FC=1.2, $p=0.002$), and six being down-regulated: miR-219-5p (FC=0.4, $p<0.0001$), miR-101a-3p (FC=0.8, $p<0.0001$), miR-33-5p (FC=0.5, $p<0.0001$), miR-

30a-5p (FC=0.8, p<0.0001), miR-136-5p (FC=0.7, p=0.002), and miR-30e-5p (FC=0.7, p=0.003) (Table 3.4).

Table 3.4. miRNA profiling results in the prefrontal cortex of F3 adults (P133)

Comparison	miRNA	Fold Change	p-value
SSSS vs. CT	rno-miR-92b-3p	**1.380**	0.000
	rno-miR-219-5p	**0.371**	0.000
	rno-miR-101a-3p	**0.752**	0.000
	rno-miR-33-5p	**0.465**	0.000
	rno-miR-92a-3p	**1.693**	0.000
	rno-miR-30a-5p	**0.761**	0.000
	rno-miR-128-3p	**1.223**	0.002
	rno-miR-136-5p	**0.701**	0.002
	rno-miR-30e-5p	**0.668**	0.003
SNNN vs. CT	rno-miR-92b-3p	**1.411**	0.000
SSSS vs. SNNN	rno-miR-219-2-3p	**0.674**	0.000
	rno-miR-338-3p	**0.652**	0.000
	rno-miR-219-5p	**0.376**	0.000
	rno-miR-21-5p	**0.752**	0.002

Overall, the expression of 14 miRNAs was altered in P133 female prefrontal cortex in the F3 generation. To verify whether changes in the miRNA patterns in P133 prefrontal cortex were similar to E21 cortex and placenta expression, we plotted fold changes of each miRNA expression in adult P133 prefrontal cortex, E21 pup cortex and placenta (Fig. 3.9). For more details on the miRNA expression data of each miRNA see Table S5. Most of the miRNAs that were altered in P133 cortex in the F3 generation are broadly conserved (see Table 3.5).

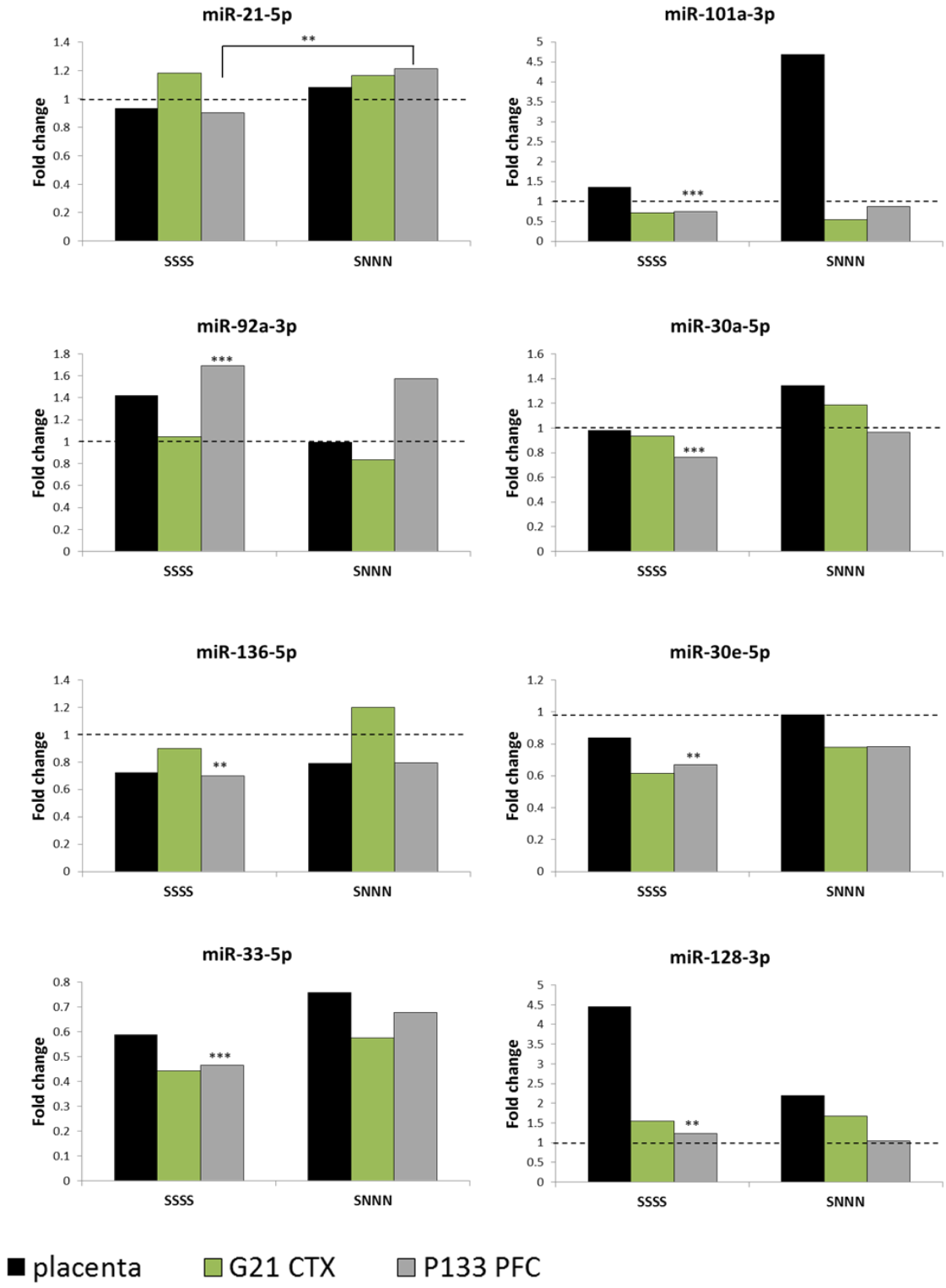


Figure 3.9. MiRNA expression in placenta (black), E21 embryonic cortex (green), and P133 prefrontal cortex (grey). Data is represented as fold change from controls. Asterisks indicate statistical significance *p≤0.05; **p≤0.01; ***p≤0.001, calculated using DESeq bioconductor package. Sample size was n=3.

Table 3.5. Evolutionary conservation of altered miRNAs.

evolutionary conservation	miRNA
broadly conserved miRNA	rno-miR-92b-3p
	rno-miR-219-5p
	rno-miR-101a-3p
	rno-miR-33-5p
	rno-miR-92a-3p
	rno-miR-30a-5p
	rno-miR-128-3p
	rno-miR-30e-5p
	rno-miR-92b-3p
	rno-miR-219-2-3p
	rno-miR-338-3p
	rno-miR-219-5p
	rno-miR-21-5p
conserved	rno-miR-136-5p

Analysis of validated miRNA targets in the F3 generation

E21 cortex and placenta

Analysis of miRNA targets, using the miRecords database

Analysis of validated miRNA targets of miR-100 and miR-99b was done using the MiRecord database. The results are summarized in Table 3.6. Based on these data, FGFR3, IGF-IR, TGF-β, mTOR were chosen for further analysis of protein expression.

Table 3.6. Validated targets of miRNAs unique for the SNNN group (using miRecord database).

miRNA	validated targets
miR-100	mTOR, IGF-IR, RPTOR, SNF2H, BMPR2, FGFR3, PLK1, IGF-2
miR-99b	RAVER2, FGFR3, TGF-β

Protein expression analysis of miRNA targets, using Western Immunoblotting

We analysed expression of four validated targets of miR-100 and miR-99b, including FGFR3, IGF-IR, TGF-β, and mTOR in both placenta and embryonic cortex in the offspring of the F3 generation. These targets were chosen because of their important role in growth and

development. The results of Western Immunoblotting are summarised in Figs. 3.10 – 3.11, S12-15.

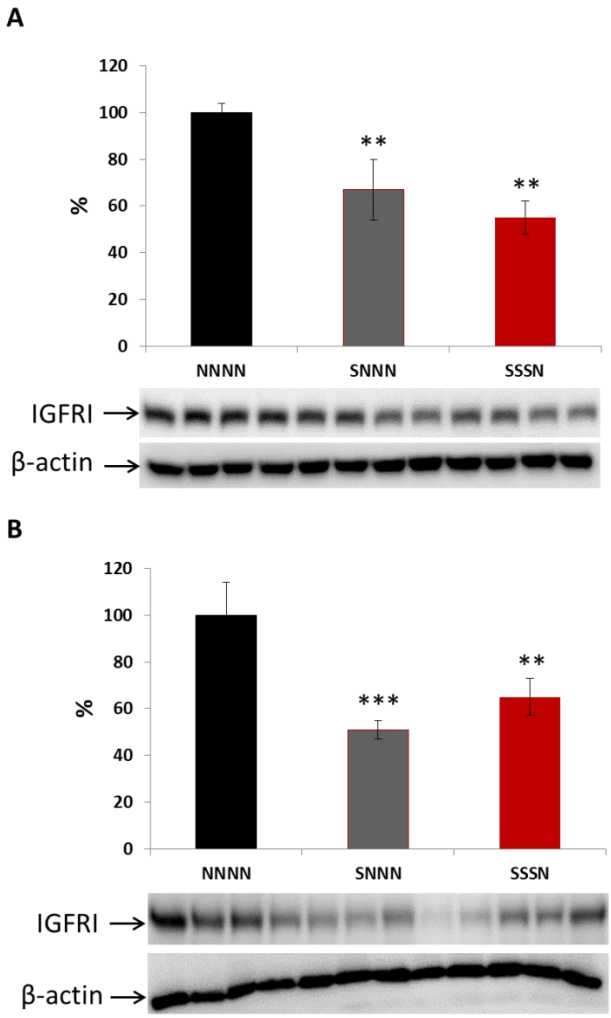


Figure 3.10. IGFRI protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation. Asterisks indicate statistical significance (**p≤0.01, ***p≤0.001; n=4).

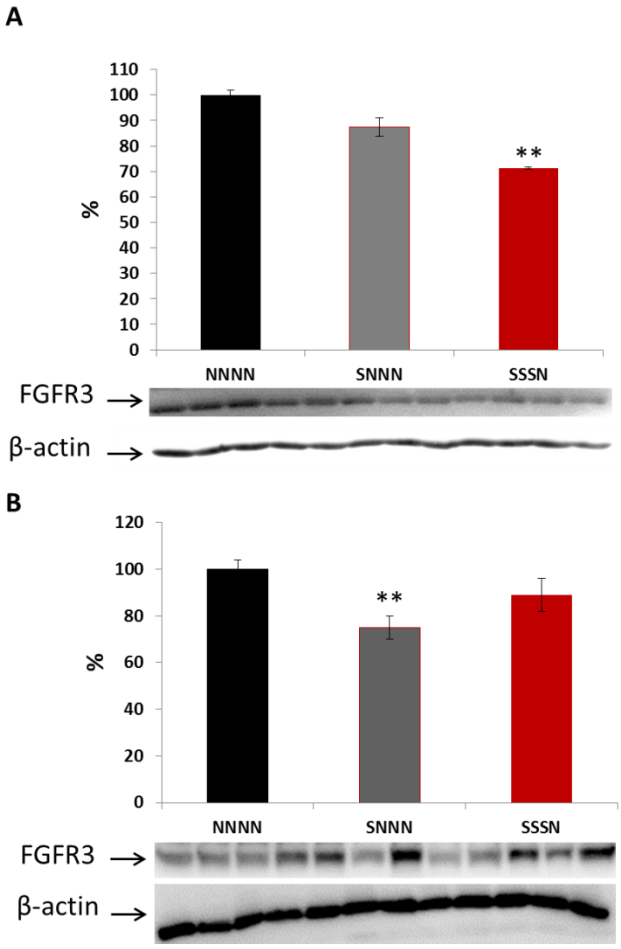


Figure 3.11. FGFR3 protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation. Asterisks indicate statistical significance (**p≤0.01; n=4).

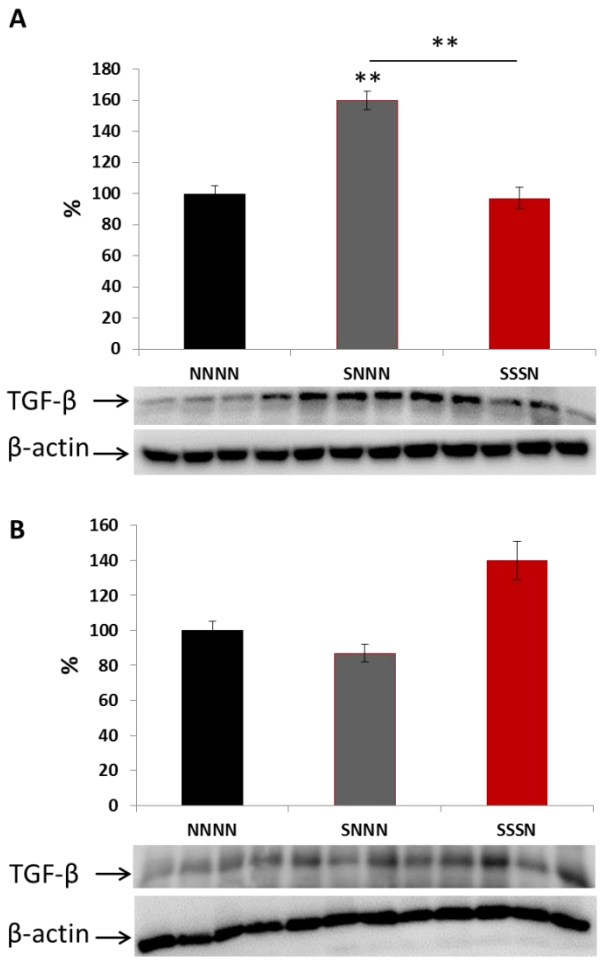


Figure 3.12. TGF-β protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation. Asterisks indicate statistical significance (**p≤0.01; n=4).

We also checked the expression of some proteins important for the stress response, epigenetic regulation and neurodevelopment, such as BDNF, DNMT3a and GR, however no significant changes were found (Supplemental materials, Figs. S12 – S14).

P133 prefrontal cortex miRNA pathways analysis

To identify molecular pathways potentially altered by the expression of multiple microRNAs, DIANA-miRpath v.2.0 was used (Vlachos et al 2012). The lists of miRNAs used in this analysis and the number of validated targets for each of the miRNAs available in the DIANA-miRpath database are shown in the Table 3.7.

Table 3.7. Lists of miRNAs altered in different comparisons

Comparison	Tissue	List of miRNAs	# of miRNA targets
NNNN vs. NNN	placenta	miR-871*	n/a
		miR-134	n/a
		miR-154	n/a
SSN vs. NNN	placenta	miR-409-5p	n/a
		miR-9	714
		miR-29a	n/a
		miR-493*	n/a
		miR-3596d	n/a
		miR-331	3
SSSN vs. NNNN	placenta	miR-99b	43
		miR-322*	n/a
		miR-30e*	17
		miR-331	3
		miR-409-5p	n/a
		miR-154	1
		miR-871*	n/a
	G21 cortex	miR-409-5p	n/a
		miR-331	3
SNNN vs. NNNN	placenta	miR-871*	n/a
		miR-322*	n/a
		miR-331	3
		miR-409-5p	n/a
		miR-675*	n/a
		miR-100	231
		miR-370	4
		miR-501*	7
		miR-153	9
		miR-181a	685
		miR-99b	43
	G21 cortex	miR-331	3
		miR-409-5p	n/a
		miR-100	231
		miR-99b	43
SSSS vs. NNNN	P133 prefrontal cortex	miR-92b-3p	13
		miR-219-5p	n/a
		miR-101a-3p	8
		miR-33-5p	4
		miR-92a-3p	958

		miR-30a-5p	457
		miR-128-3p	297
		miR-136-5p	275
		miR-30e-5p	394
SNNN vs. NNNN		miR-92b-3p	13
SNNN vs. SSSS		miR-219-2-3p	n/a
		miR-338-3p	10
		miR-219-5p	2
		miR-21-5p	581

Placenta

The analysis of the list of miRNAs changed in SNNN vs. CT comparison in placenta showed 14 KEGG pathways that may be altered due to the observed changes in miRNA expression (Table 3.8).

Table 3.8. KEGG pathways for the list of miRNAs changed in SNNN vs. CT in placenta

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Pathways in cancer	0.000009	13	4	miR-100,-153, -181a, -99b
Long-term depression	0.000069	4	2	miR-181a, -99b
Prostate cancer	0.00034	6	2	miR-181a, -153
Wnt signaling pathway	0.000711	9	2	miR-181a, -99b
PI3K-Akt signaling pathway	0.000752	12	3	miR-100,-153, -181a
Notch signaling pathway	0.002648	4	2	miR-181a, -99b
Melanogenesis	0.004133	6	2	miR-181a, -99b
Adherens junction	0.004561	5	2	miR-181a, -99b
Cell cycle	0.004561	7	3	miR-181a, -99b, -100
TGF-beta signaling pathway	0.005987	5	3	miR-181a, -99b, -100
Transcriptional misregulation in cancer	0.014155	7	3	miR-370, -153, -181a
Small cell lung cancer	0.017646	4	2	miR-181a, -153

For the pathways details see Supplemental materials Figure S1-S14.

Pathways union analysis for the list of miRNAs changed in placenta in SNNN vs. CT showed 3 KEGG pathways being altered (see Table 3.9). For the hit map visualization of the hierarchical clustering results see Fig. 3.13.

Table 3.9. Pathways union SNNN vs CT in placenta.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Pathways in cancer	0.002	11	2	miR-153, -181a
PI3K-Akt signaling pathway	0.02	12	3	miR-100, -153, -181a
Prostate cancer	0.03	6	2	miR-153, -181a

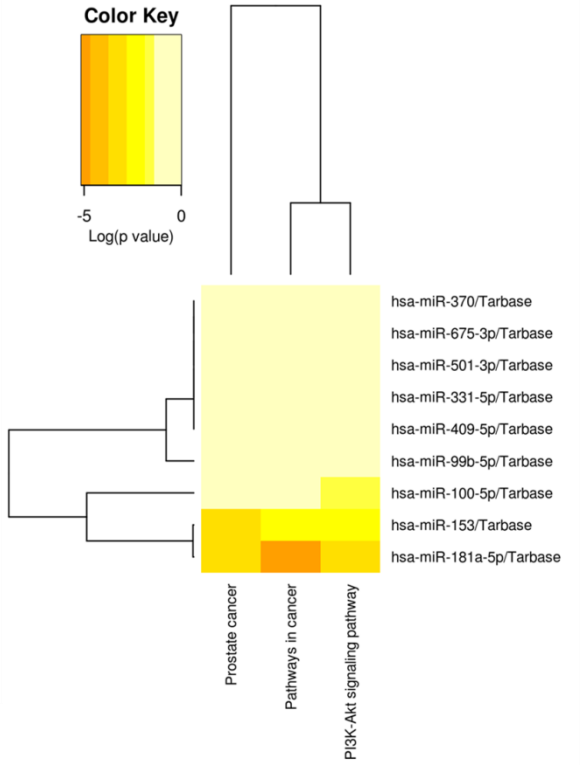


Figure 3.13. MiRNA vs. pathways heat map (SNNN vs CT, placenta). Clustering is based on the p-value significance levels. Darker colours represent lover p-values (Papadopoulos et al 2009).

The analysis of the list of miRNAs changed in SSSN vs. CT comparison in placenta showed 16 KEGG pathways that may be altered due to observed changes in the miRNA expression (Table 3.10).

Table 3.10. KEGG pathways for the list of miRNAs changed in SSSN vs. CT group in placenta.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Circadian entrainment	0.000004	3	2	miR-99b, -30e*
Morphine addiction	0.000004	3	2	miR-99b, -30e*

Aminoacyl-tRNA biosynthesis	0.001068	3	2	miR-99b, -30e*
Phototransduction	0.001068	2	2	miR-99b, -30e*
Dopaminergic synapse	0.001523	4	2	miR-99b, -30e*
Neurotrophin signaling pathway	0.015129	3	2	miR-99b, -30e*
Cholinergic synapse	0.0219	2	2	miR-99b, -30e*
Retrograde endocannabinoid signaling	0.02359	2	2	miR-99b, -30e*

KEGG pathway with the highest number of altered genes is shown in Supplemental materials (Fig. S17).

Pathways union analysis for the list of miRNAs changed in placenta in SSSN vs. CT showed two KEGG pathways being altered (Table 3.11).

Table 3.11. Pathways union SSSN vs CT in placenta.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Circadian entrainment	0.003	3	2	miR-99b, -30e*
Morphine addiction	0.003	3	2	miR-99b, -30e*

E21 cortex

Similar analysis was done for the list of miRNAs altered in cortex. DIANA-miRpath analysis revealed that 16 KEGG pathways may be altered due to changes in miRNA expression in SNNN vs. CT comparison in E21 cortex (Table 3.12).

Table 3.12. KEGG pathways for the list of miRNAs altered in SNNN vs. CT group in cortex.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Pertussis	0.001536	3	1	miR-99b
Spliceosome	0.004183	2	1	miR-99b
Axon guidance	0.005186	2	1	miR-99b
Circadian entrainment	0.014137	2	1	miR-99b
Oocyte meiosis	0.014137	2	2	miR-99b, -100
Regulation of actin cytoskeleton	0.015616	3	2	miR-99b, -100
Gastric acid secretion	0.018667	2	1	miR-99b
Progesterone-mediated oocyte maturation	0.024912	2	2	miR-99b
Cell cycle	0.040825	2	2	miR-99b, -100
TGF-beta signaling pathway	0.040825	2	2	miR-99b, -100

Melanogenesis	0.041808	2	1	miR-99b
Leukocyte transendothelial migration	0.046663	2	1	miR-99b

The pathways union analysis for the list of miRNAs changed in cortex in SNNN vs. CT showed 12 KEGG pathways being altered (Table 3.13).

Table 3.13. Pathways union analysis for the list of miRNAs altered in SNNN vs. CT group in cortex.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Oocyte meiosis	0.000944	1	1	miR-100
Pertussis	0.005313	3	1	miR-99b
Spliceosome	0.01184	2	1	miR-99b
Axon guidance	0.014417	2	1	miR-100
Circadian entrainment	0.039804	2	1	miR-99b

P133 prefrontal cortex

The analysis of P133 prefrontal cortex revealed that 22 KEGG pathways may be altered due to changes in miRNA expression in the SNNN vs. CT comparison (Table 3.14).

Table 3.14. KEGG pathways for the list of miRNAs altered in SSSS vs. CT group in P133 prefrontal cortex.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.000000	10	2	miR-92a-3p, -30a-5p
RNA transport	0.000000	18	4	miR-92a-3p, -30a-5p, -92b-3p, -30e-5p
Protein processing in endoplasmic reticulum	0.000000	16	3	miR-92a-3p, -30a-5p, -30e-5p
Colorectal cancer	0.00021	8	2	miR-92a-3p, -30a-5p
mRNA surveillance pathway	0.00042	10	2	miR-92a-3p, -30a-5p
Amyotrophic lateral sclerosis (ALS)	0.00205	7	1	miR-30a-5p
Ribosome biogenesis in eukaryotes	0.00511	8	2	miR-30a-5p, -30e-5p
Protein export	0.00538	4	1	miR-30a-5p
Adherens junction	0.00596	8	2	miR-92a-3p, -30a-5p
Valine, leucine and isoleucine biosynthesis	0.00741	1	1	miR-92a-3p
PI3K-Akt signaling pathway	0.00741	21	3	miR-92a-3p, -30a-5p, -30e-5p

Nucleotide excision repair	0.00825	5	2	miR-30a-5p, -30e-5p
Purine metabolism	0.01137	11	2	miR-30a-5p, -30e-5p
Glutathione metabolism	0.01137	5	2	miR-30a-5p, -30e-5p
Ubiquitin mediated proteolysis	0.01278	11	3	miR-92a-3p, -30a-5p, -30e-5p
Focal adhesion	0.01361	14	2	miR-92a-3p, -30a-5p
RNA degradation	0.01405	7	2	miR-92a-3p, -30a-5p
Oocyte meiosis	0.01523	11	3	miR-92a-3p, -30a-5p, -219-5p
Hypertrophic cardiomyopathy (HCM)	0.0172	7	2	miR-92a-3p, -30a-5p
Citrate cycle (TCA cycle)	0.03409	3	1	miR-30a-5p
Vitamin B6 metabolism	0.03409	1	1	miR-30a-5p
Long-term potentiation	0.04375	6	2	miR-30a-5p, -219-5p

The pathways union analysis for the list of miRNAs changed in cortex in SSSS vs. CT showed 9 KEGG pathways being altered (see Table 3.15). For the hit map visualization of the hierarchical clustering results see Fig. 3.14. KEGG pathway for arrhythmogenic right ventricular cardiomyopathy is shown in Supplemental materials (Fig. S20).

Table 3.15. Pathways union analysis for the list of miRNAs altered in SSSS vs. CT group in P133 prefrontal cortex.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.000004	9	1	miR-30a-5p
RNA transport	0.000005	15	3	miR-92b-3p, -30a-5p, -30e-5p
Base excision repair	0.000030	3	2	miR-30a-5p, -30e-5p
Colorectal cancer	0.00091	8	2	miR-92a-3p, -30a-5p
Protein processing in endoplasmic reticulum	0.00191	16	2	miR-92a-3p, -30a-5p
Circadian entrainment	0.00834	2	2	miR-30e-5p, -219-5p
TGF-beta signaling pathway	0.00939	4	1	miR-92a-3p
Adherens junction	0.01083	4	1	miR-92a-3p

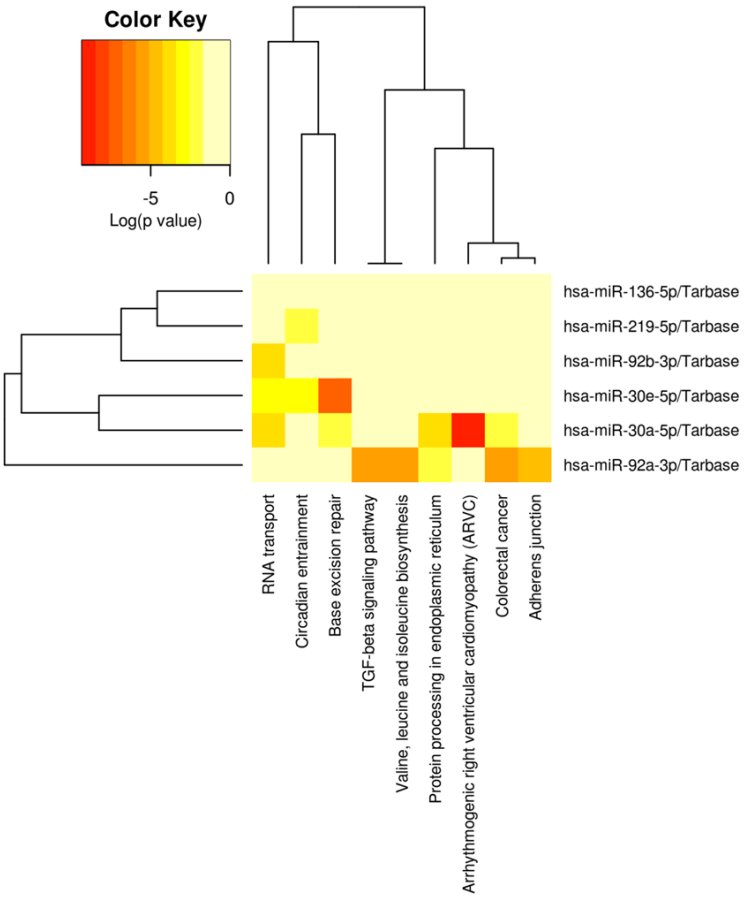


Figure 3.14. MiRNA vs. pathways heat map (SSSS vs. CT, P133 prefrontal cortex). Clustering is based on the p-value significance levels. Darker colours represent lower p-values.

DIANA-miRpath analysis of the list of miRNAs changed in SNNN vs. SSSS comparison in P133 prefrontal cortex revealed that 39 KEGG pathways may be altered due to changes in miRNA expression (Table 3.16, Fig. 3.15).

Table 3.16. KEGG pathways for the list of miRNAs altered in SNNN vs. SSSS group in P133 prefrontal cortex.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Pathways in cancer	6.77E-15	30	2	miR-338-3p, -21-5p
Colorectal cancer	8.03E-13	12	1	miR-21-5p
Hepatitis B	1.42E-10	17	1	miR-21-5p
Bladder cancer	1.58E-09	9	2	miR-338-3p, -21-5p
Pancreatic cancer	6.82E-08	11	1	miR-21-5p
p53 signaling pathway	1.25E-05	9	1	miR-21-5p
HIF-1 signaling pathway	4.97E-05	11	2	miR-21-5p, -219-5p
ErbB signaling pathway	8.45E-05	7	2	miR-21-5p, -219-5p

Small cell lung cancer	8.63E-05	9	1	miR-21-5p
Endometrial cancer	0.000101	7	1	miR-21-5p
Chronic myeloid leukemia	0.000124	8	1	miR-21-5p
Prostate cancer	0.000175	9	1	miR-21-5p
MAPK signaling pathway	0.000689	17	2	miR-338-3p, -21-5p
GnRH signaling pathway	0.000689	8	3	miR-338-3p, -21-5p, -219-5p
Lysine degradation	0.001987	5	1	miR-21-5p
Wnt signaling pathway	0.003386	11	2	miR-21-5p, -219-5p
TGF-beta signaling pathway	0.005446	8	1	miR-21-5p
Glycerophospholipid metabolism	0.005446	9	2	miR-338-3p, -21-5p
mTOR signaling pathway	0.005887	6	1	miR-21-5p
Glioma	0.007809	6	2	miR-21-5p, -219-5p
Sphingolipid metabolism	0.00921	4	1	miR-21-5p
Synthesis and degradation of ketone bodies	0.011171	2	1	miR-21-5p
Focal adhesion	0.014249	12	1	miR-21-5p
Herpes simplex infection	0.015586	12	1	miR-21-5p
Fc gamma R-mediated phagocytosis	0.015858	7	2	miR-338-3p, -21-5p
Fanconi anemia pathway	0.015871	5	1	miR-21-5p
Melanoma	0.016797	6	1	miR-21-5p
Amyotrophic lateral sclerosis (ALS)	0.016885	5	1	miR-21-5p
Basal cell carcinoma	0.018585	5	2	miR-338-3p, -21-5p
Chagas disease (American trypanosomiasis)	0.03061	7	1	miR-21-5p
Basal transcription factors	0.03274	4	1	miR-21-5p
Circadian rhythm	0.037252	3	1	miR-21-5p
Hypertrophic cardiomyopathy (HCM)	0.037943	6	1	miR-21-5p
Mismatch repair	0.039164	2	1	miR-21-5p
VEGF signaling pathway	0.039164	5	2	miR-338-3p, -21-5p
Non-small cell lung cancer	0.042265	5	1	miR-21-5p
Butanoate metabolism	0.043175	3	1	miR-21-5p
Endocytosis	0.046497	10	1	miR-21-5p

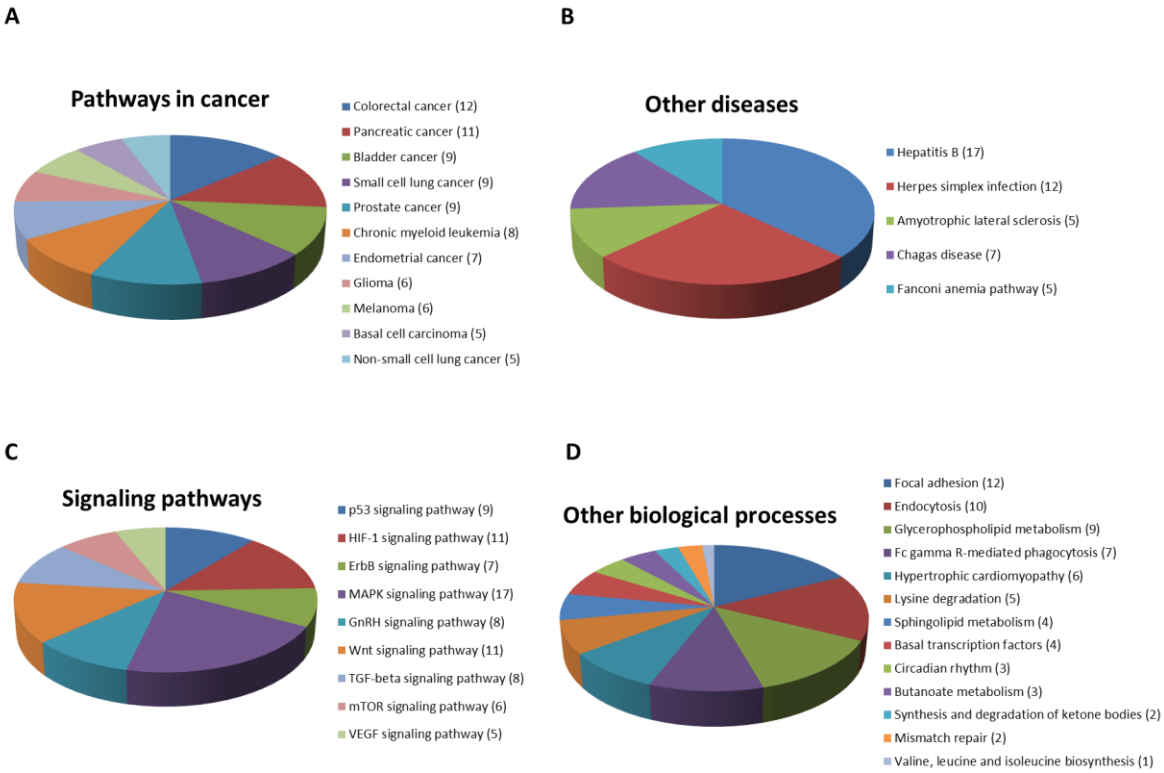


Figure 3.15. KEGG pathways potentially altered by the changes in the miRNA expression in P133 prefrontal cortex (SNNN vs. SSSS). The number of target genes that are part of the pathway is indicated in brackets.

The pathways union analysis for the list of miRNAs changed in cortex in SNNN vs. SSSS showed 17 KEGG pathways being altered (Table 3.17). For the hit map visualization of the hierarchical clustering results see Fig. 3.16.

Table 3.17. Pathways union analysis for the list of miRNAs altered in SNNN vs. SSSS group in P133 prefrontal cortex.

KEGG pathway	p-value	# of genes	# of miRNAs	miRNAs
Pathways in cancer	0.000000	30	2	miR-338-3p, -21-5p
Colorectal cancer	0.000000	12	1	miR-21-5p
Hepatitis B	0.000000	17	1	miR-21-5p
Bladder cancer	0.000001	9	2	miR-338-3p, -21-5p
Pancreatic cancer	0.000031	11	1	miR-21-5p
GnRH signaling pathway	0.000427	3	2	miR-338-3p, -21-5p
HIF-1 signaling pathway	0.001394	11	2	miR-21-5p, -219-5p
p53 signaling pathway	0.002128	9	1	miR-21-5p
ErbB signaling pathway	0.00239	7	2	miR-21-5p, -219-5p

Small cell lung cancer	0.011305	9	1	miR-21-5p
Endometrial cancer	0.01364	7	1	miR-21-5p
Chronic myeloid leukemia	0.01462	8	1	miR-21-5p
Prostate cancer	0.018142	9	1	miR-21-5p
MAPK signaling pathway	0.020843	16	1	miR-21-5p
Glioma	0.032244	6	2	miR-21-5p, -219-5p
Wnt signaling pathway	0.032542	11	2	miR-21-5p, -219-5p
Glycerophospholipid metabolism	0.035192	9	2	miR-21-5p, -219-5p

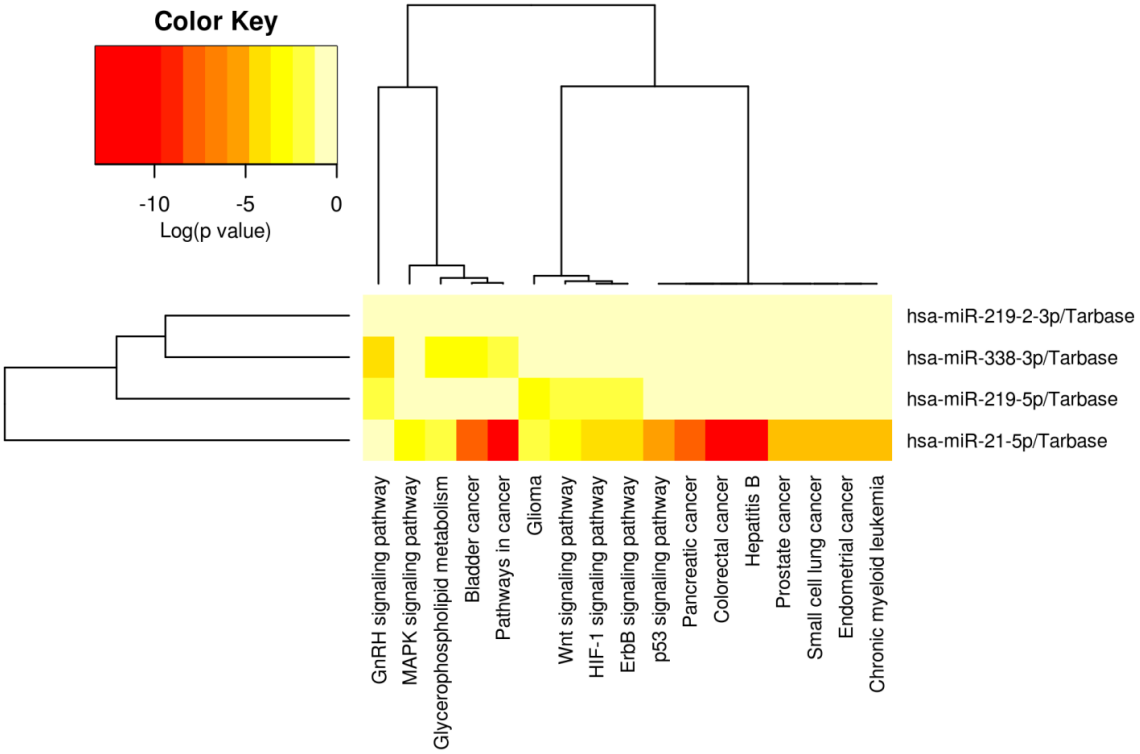
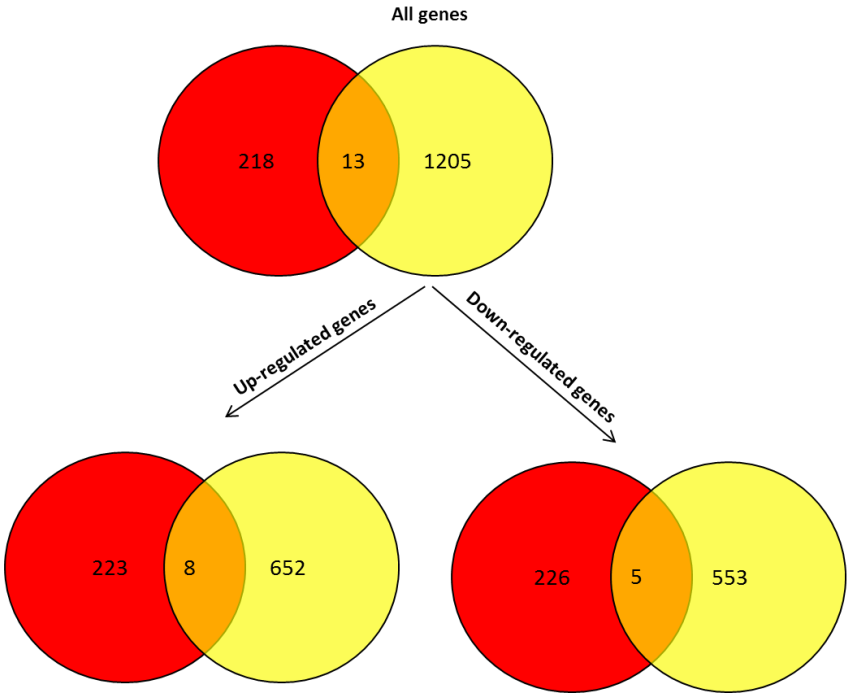


Figure 3.16. miRNA vs. pathways heat map (SNNN vs. SSSS, P133 prefrontal cortex). Clustering is based on the p-value significance levels. Darker colours represent lower p-values.

Correlation analysis: mRNA vs. miRNA expression

E21 Cortex (SNNN group)

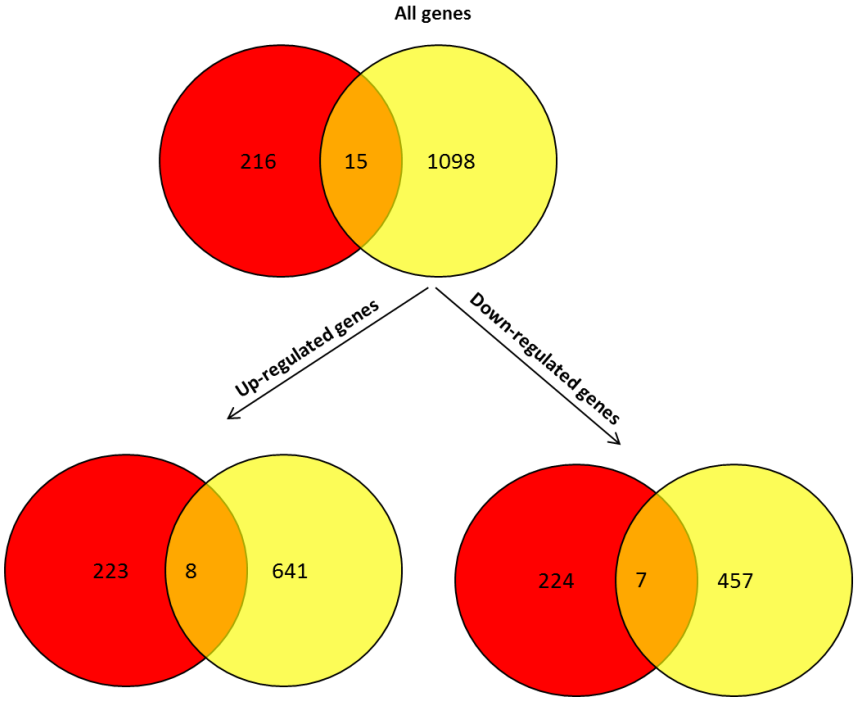
According to miRTarBase miR-100 has 231 validated targets. Thirteen targets were found to be significantly changed on mRNA level in E21 female cortex in response to stress in SNNN group (Fig. 3.17).



miR-100		
231 validated targets vs. 1218 genes in SNNN E21 cortex list		
total	up-regulated	down-regulated
13	8	5
BECN1	BECN1	ID1
HSPA14	HSPA14	RPL31
ID1	NFIA	SERBP1
NFIA	ODC1	TEX264
ODC1	PNRC1	VPS45
PNRC1	PTP4A1	
PTP4A1	RCN2	
RCN2	RPL7	
RPL31		
RPL7		
SERBP1		
TEX264		
VPS45		

Figure 3.17. Venn diagrams showing the number of genes significantly changed in E21 cortex in SNNN group that appeared to be targets of miR-100. Expression of 1218 genes was significantly altered in E21 cortex in response to stress in SNNN group (660 genes were up-regulated, whereas 558 – down-regulated). Thirteen genes out of the total 1,218 appeared to be targets of miR-100.

Fifteen targets were found to be significantly changed on mRNA level in placenta in response to stress in the SNNN group (Fig. 3.18).



miR-100		
231 validated targets vs. 1113 genes in SNNN placenta list		
total	up-regulated	down-regulated
15	8	7
APBA3	BECN1	APBA3
BECN1	CCNG1	ID1
CCNG1	CUL5	IGF1R
CUL5	HSPA14	RPLP1
HSPA14	PNRC1	RPS15
ID1	RCN2	XRCC6
IGF1R	RPL7	YWHAE
PNRC1	TCP1	
RCN2		
RPL7		
RPLP1		
RPS15		
TCP1		
XRCC6		
YWHAE		

Figure 3.18. Venn diagrams showing the number of genes significantly changed in placenta in SNNN group that appeared to be targets of miR-100. Expression of 1,113 genes was significantly altered in placentas attached to the female embryos in response to stress in SNNN group (649 genes were up-regulated, whereas 464 – down-regulated). Fifteen genes out of the total 1,113 appeared to be targets of miR-100.

According to miRTarBase miR-99b has 43 validated targets. Only one target MFN2 was found to be altered on mRNA level in both placenta and E21 cortex in SNNN group.

miR-331 has 3 validated targets according to the miRTaRBase database, none of them was found to be altered on mRNA level in response to stress in SNNN group in either placenta or E21 cortex. No available targets were found for miR-409-5p in the miRTaRBase database.

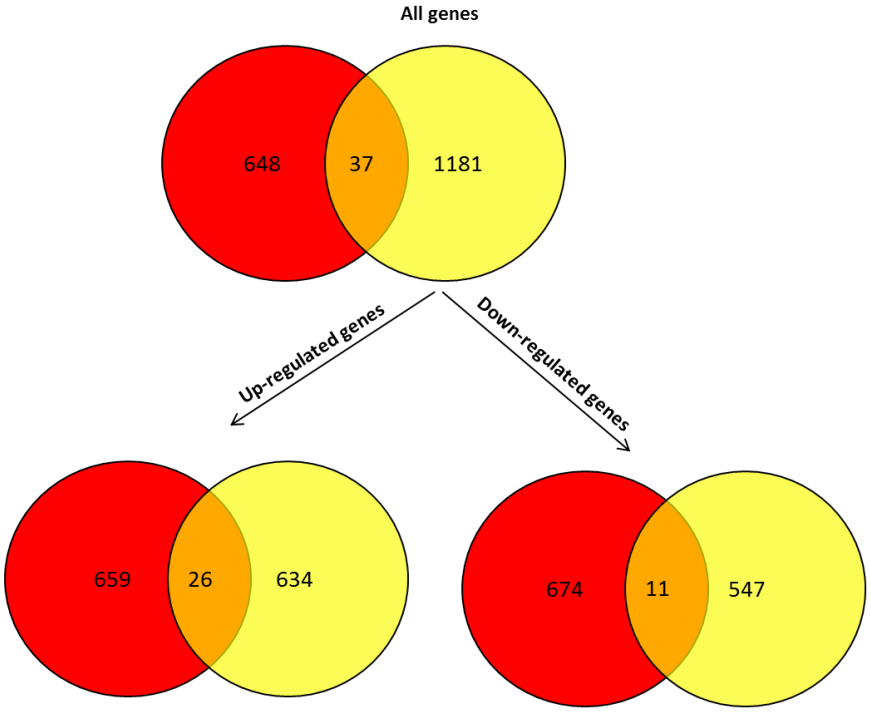
To verify whether miRNA expression changes correlate with the mRNA expression changes we performed Pearson correlation analysis (Table 3.18).

Table 3.18. Results of correlation analysis between E21 SNNN miRNA expression in cortex and mRNA expression (miRNA targets) in E21 SNNN cortex and placenta. P-values <0.05 are highlighted in red.

miRNA	Cortex			Placenta		
	mRNA	Pearson Correlation	Sig. (2-tailed)	mRNA	Pearson Correlation	Sig. (2-tailed)
miR-99b	Mfn2	.930	.007	Mfn2	.911	.012
miR-100	Becn1	-.792	.060	Apba3	.802	.055
	Hspa14	-.913	.011	Becn1	-.893	.016
	Id1	.808	.052	Ccng1	-.963	.002
	Nfia	-.923	.009	Cul5	-.850	.032
	Odc1	-.895	.016	Hspa14	-.898	.015
	Pnrc1	-.932	.007	Id1	.792	.060
	Ptp4a1	-.871	.024	Igf1r	.924	.008
	Rcn2	-.947	.004	Pnrc1	-.838	.037
	Rpl31	.908	.012	Rcn2	-.912	.011
	Rpl7	-.879	.021	Rpl7	-.902	.014
	Serbp1	.910	.012	Rplp1	.838	.037
	Tex264	.926	.008	Rps15	.941	.005
	Vps45	.861	.028	Tcp1	-.915	.010
				Xrcc6	.759	.080
				Ywhae	.931	.007

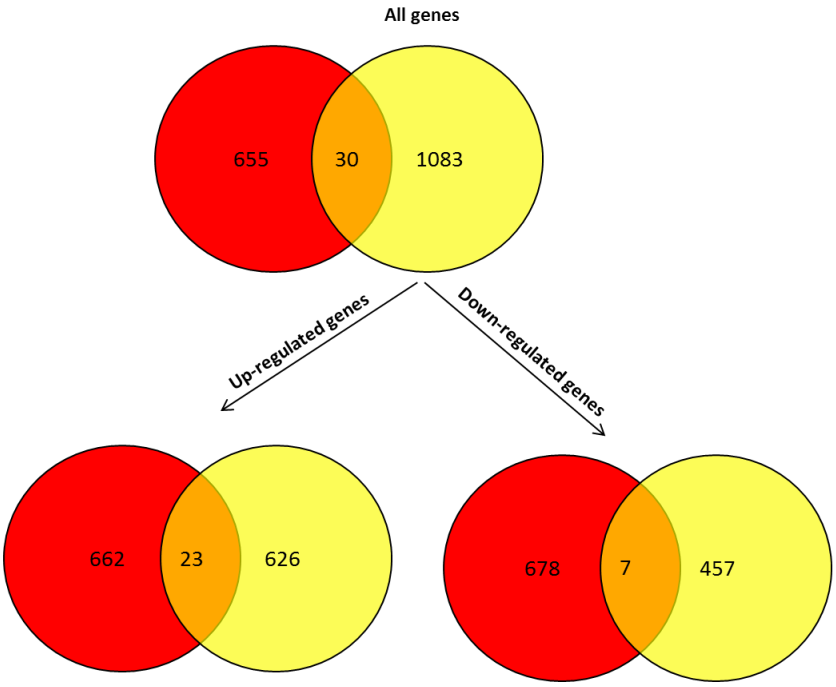
Placenta (SNNN group)

Out of 11 miRNAs that were altered in response to stress in placentas of the SNNN group, only 5 miRNAs were referenced in the miRTarBase database, including miR-331, miR-100, miR-99b, miR-501*, miR-181. Correlation analysis of miR-331, -100, and -99b was covered in the previous section. The miRTarBase database showed 7 validated targets for miR-501*. None of them was found to be altered on the mRNA level in response to stress in the SNNN group in either placenta or E21 cortex. According to the miRTaRBase, miR-181a has 685 targets. 37 targets were found to be significantly changed on mRNA level in E21 female cortex in response to stress in SNNN group (Fig. 3.19), whereas 30 targets significantly changed in placenta (Fig. 3.20).



miR-181a		
685 validated targets vs. 1218 genes in SNNN E21 cortex list		
total	up-regulated	down-regulated
37	26	11
ACSL4	ACSL4	AKAP12
AKAP12	ARL6IP6	AP1M1
AP1M1	CDKN1B	ARF2
ARF2	DNAJC7	BPGM
ARL6IP6	EDNRB	KIFC2
BPGM	ENAH	NDRG2
CDKN1B	EPHA5	OGT
DNAJC7	FBXO11	PCNX
EDNRB	GPM6A	PSAP
ENAH	GRIA2	PTPRD
EPHA5	GRIA3	SPRY2
FBXO11	KRAS	
GPM6A	MCL1	
GRIA2	MYO5A	
GRIA3	NFIA	
KIFC2	NID1	
KRAS	NPTN	
MCL1	NRP1	
MYO5A	PABPC1	
NDRG2	PDIA3	
NFIA	RALA	
NID1	SSR3	
NPTN	TCF4	
NRP1	TMEM135	
OGT	USP33	
PABPC1	YWHAG	
PCNX		
PDIA3		
PSAP		
PTPRD		
RALA		
SPRY2		
SSR3		
TCF4		
TMEM135		
USP33		
YWHAG		

Figure 3.19. Venn diagrams showing the number of genes significantly changed in E21 cortex in the SNNN group that appeared to be targets of miR-181a. Thirty-seven genes out of the total 1,218 appeared to be targets of miR-181a.



miR-181a		
685 validated targets vs. 1113 genes in SNNN placenta list		
total	up-regulated	down-regulated
30	23	7
ACSL4	ACSL4	AP1M1
AP1M1	ARL6IP6	DDIT4
ARL6IP6	CCNG1	FOS
CCNG1	CDKN1B	GADD45G
CDKN1B	DUSP6	KLF6
DDIT4	EDNRB	PPP3R1
DUSP6	ENAH	PSAP
EDNRB	FBXO11	
ENAH	ID2	
FBXO11	KRAS	
FOS	MAP2K1	
GADD45G	MCL1	
ID2	NPTN	
KLF6	NUPL1	
KRAS	PABPC1	
MAP2K1	PDIA3	
MCL1	PPP1CB	
NPTN	RALA	
NUPL1	SSR3	
PABPC1	SV2B	
PDIA3	TMEM135	
PPP1CB	TSN	
PPP3R1	YWHAG	
PSAP		
RALA		
SSR3		
SV2B		
TMEM135		
TSN		
YWHAG		

Figure 3.20. Venn diagrams showing the number of genes significantly changed in placenta in the SNNN group that appeared to be targets of miR-181a. Thirty genes out of the total 1,113 appeared to be targets of miR-181a.

To verify whether expression changes in miR-181a in placenta in the SNNN group correlate with the mRNA expression changes of miR-181a targets in both placenta and embryonic cortex we performed Pearson correlation analysis (Table 3.19). Our results showed that miR-181a expression significantly ($p<0.05$) correlated with the mRNA expression of 20 miRNA targets (Table 3.19).

Table 3.19. Results of correlation analysis of miR-181 expression in SNNN group in placenta vs. E21 SNNN cortex and placenta mRNA (miRNA targets).

mRNA	Pearson Correlation	Sig. (2-tailed)	mRNA	Pearson Correlation	Sig. (2-tailed)
Cortex			Placenta		
Acsl4	-.832	.040	Acsl4	-.675	.141
Akap12	.892	.017	Ap1m1	.720	.107
Ap1m1	.804	.054	Arl6ip6	-.813	.049

Arf2	.781	.067	Ccng1	-.892	.017
Arl6ip6	-.682	.135	Cdkn1b	-.893	.017
Bpgm	.725	.103	Ddit4	.650	.162
Cdkn1b	-.710	.114	Dusp6	-.565	.242
Dnajc7	-.964	.002	Ednrb	-.866	.026
Ednrb	-.826	.043	Enah	-.642	.169
Enah	-.787	.063	Fbxo11	-.780	.067
Epha5	-.694	.126	Fos	.685	.134
Fbxo11	-.883	.020	Gadd45g	.896	.016
Gpm6a	-.753	.084	Id2	-.656	.157
Gria2	-.545	.263	Klf6	.857	.029
Gria3	-.700	.122	Kras	-.699	.122
KIFC2	.592	.216	Map2k1	-.606	.202
Kras	-.547	.261	Mcl1	-.575	.233
Mcl1	-.740	.093	Nptn	-.773	.071
Myo5a	-.684	.134	Nupl1	-.689	.130
Ndrg2	.753	.084	Pabpc1	-.841	.036
Nfia	-.797	.058	Pdia3	-.820	.046
Nid1	-.738	.094	Ppp1cb	-.640	.171
Nptn	-.789	.062	Ppp3r1	.698	.123
Nrp1	-.806	.053	Psap	.964	.002
Ogt	.868	.025	Rala	-.746	.088
Pabpc1	-.821	.045	Ssr3	-.777	.069
Pcnx	.747	.088	Sv2b	-.865	.026
Pdia3	-.771	.073	Tmem135	-.831	.040
Psap	.711	.113	Tsn	-.537	.272
Ptprd	.771	.073	Ywhag	-.777	.069
Rala	-.911	.012			
Spry2	.894	.016			
Ssr3	-.835	.039			
Tcf4	-.779	.068			
Tmem135	-.777	.069			
Usp33	-.738	.094			
Ywhag	-.815	.048			

E21 Cortex (SSSN group)

None of validated targets of miRNAs that significantly changed in E21 cortex of the offspring in the SSSN group were altered in mRNA expression analysis.

Placenta (SSSN group)

The number of genes significantly changed in placenta and embryonic cortex of the offspring in the SSSN group that appeared to be targets of altered miRNAs is summarized in Table 3.20.

Table 3.20. Validated miRNA targets that were significantly altered in placenta and embryonic cortex of the female offspring in the SSSN group.

miR-30e*: 17 targets					
from SSSN female E21 CTX list			from SSSN placenta list		
total	up	down	total	up	down
3	**2**	**1**	**2**	**1**	**1**
PPP2R2B	PPP2R2B	RPS3	RBBP7	RBBP7	YWHAE
RBBP7	RBBP7		YWHAE		
RPS3					

miR-99b: 43 targets					
from SSSN E21 CTX list			from SSSN placenta list		
total	up	down	total	up	down
1	**0**	**1**	**1**	**0**	**1**
MFN2		MFN2	MFN2		MFN2

miR-331: 3 targets					
from SSSN E21 CTX list			from SSSN placenta list		
total	up	down	total	up	down
0	**0**	**0**	**1**	**0**	**1**
			SAFB		SAFB

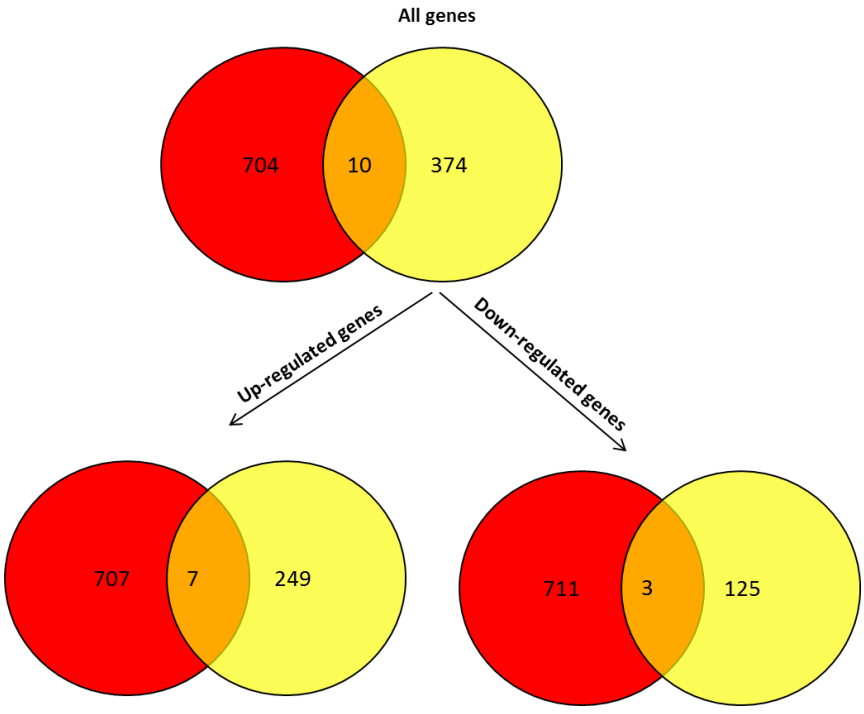
To verify whether expression changes in miR-30e*, -99b, and -331 in placenta in the SSSN group correlate with the mRNA expression changes of the corresponding miRNA targets in both placenta and embryonic cortex we performed Pearson correlation analysis (Table 3.21). Our results showed that expression of miR-99b and miR-30e* correlated significantly with the expression of their mRNA targets in both placenta and embryonic cortex; in contrast, no correlation between miR-331 and its target *Safb* was found (Table 3.21).

Table 3.21. Results of correlation analysis of miR-99b, -331, -30e* expression in SSSN group in placenta vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 generation female offspring in SSSN group.

miRNA	Cortex			Placenta		
	mRNA	Pearson Correlation	Sig. (2-tailed)	mRNA	Pearson Correlation	Sig. (2-tailed)
miR-99b	Mfn2	.985	.000	Mfn2	.889	.018
miR-331	Safb	-.612	.197	Safb	-.798	.057
miR-30e*	Ppp2r2b	-.930	.007	Rbbp7	-.940	.005
	Rbbp7	-.935	.006	Ywhae	.893	.016
	Rps3	.867	.025			

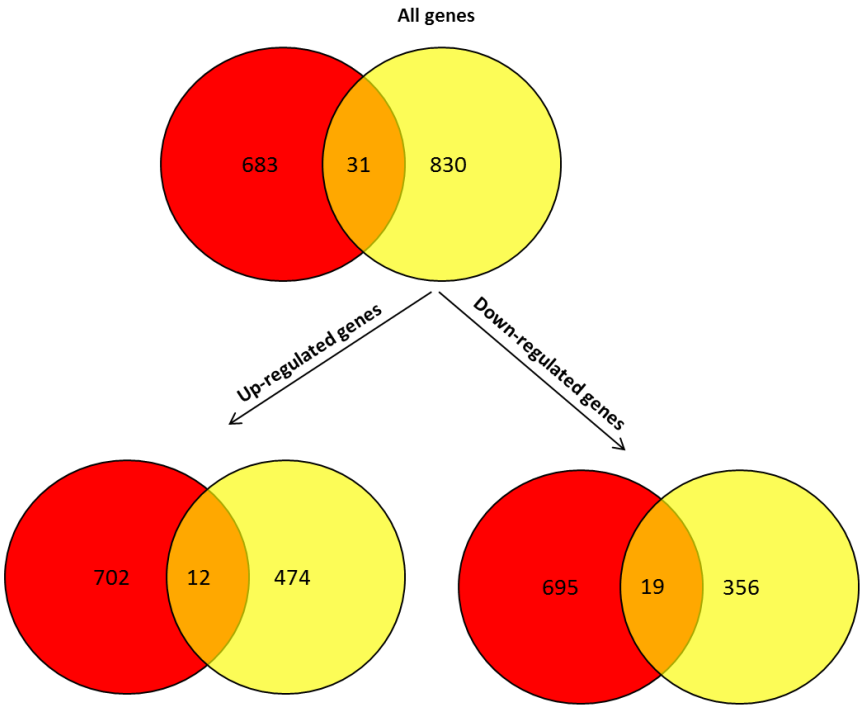
Placenta (SSN group)

Venn diagrams illustrating the number of genes significantly altered in placenta and E21 cortex of the F2 offspring in SSN group that appeared to be targets of miR-9 are shown in Fig.3.21-3.22.



miR-9		
714 validated targets vs. 384 genes in SSN E21 cortex list		
total	up-regulated	down-regulated
10	7	3
ATP6V0A1	FOXG1	ATP6V0A1
FBN2	MYO5A	FBN2
FOXG1	PAIP2	PTPRD
MYO5A	PGRMC1	
PAIP2	SERPINI1	
PGRMC1	SYAP1	
PTPRD	VIM	
SERPINI1		
SYAP1		
VIM		

Figure 3.21. Venn diagrams showing the number of genes significantly changed in E21 cortex in the SSN group that appeared to be targets of miR-9. Expression of 384 genes was significantly altered in E21 female cortex in response to stress in SSN group (256 genes were up-regulated, whereas 128 were down-regulated). Ten genes out of the total 384 appeared to be targets of miR-9.



miR-9		
714 validated targets vs. 861 genes in SSN placenta list		
total	up-regulated	down-regulated
31	12	19
ACTN4	BCLAF1	ACTN4
BCLAF1	CCNDBP1	CCNF
CCNDBP1	CCNG1	CDH1
CCNF	DHCR24	COLEC12
CCNG1	DUSP6	EIF1A
CDH1	GUCY1B3	FAM101B
COLEC12	ID2	GRN
DHCR24	ITGB3BP	IGF2
DUSP6	ITM2C	MAEA
EIF1A	PGRMC1	MYH9
FAM101B	RASA1	OPTN
GRN	VIM	PADI2
GUCY1B3		PQLC3
ID2		PRNP
IGF2		SMARCD2
ITGB3BP		SYAP1
ITM2C		SYPL1
MAEA		TAGLN
MYH9		VPS37A
OPTN		
PADI2		
PGRMC1		
PQLC3		
PRNP		
RASA1		
SYAP1		
SYPL1		
TAGLN		
VIM		
VPS37A		
SMARCD2		

Figure 3.22. Venn diagrams showing the number of genes significantly changed in placenta in the SSN group that appeared to be targets of miR-9. Expression of 861 genes was significantly altered in placenta in response to stress in the SSN group (486 genes were up-regulated, whereas 375 were down-regulated). Thirty-one genes out of the total 861 appeared to be targets of miR-9.

To verify whether expression changes in miR-9 in placenta in the SSN group correlate with the mRNA expression changes of the corresponding miRNA targets in both placenta and embryonic cortex in the SSN group we performed Pearson correlation analysis (Table 3.22). Our results showed that expression of miR-9 correlated significantly with the expression of 9 out of 10 targets of this miRNA in embryonic cortex and 25 out of 30 targets in placenta (Table 3.22).

Table 3.22. Results of correlation analysis of miR-9 expression in the SSN group in placenta vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F2 female offspring in the SSN group.

miR-9					
Cortex			Placenta		
mRNA	Pearson Correlation	Sig. (2-tailed)	miRNA/mRNA	Pearson Correlation	Sig. (2-tailed)
Atp6v0a1	-.917	.010	Actn4	-.915	.010
Fbn2	-.659	.155	Bclaf1	.822	.045
Foxg1	.987	.000	Ccndbp1	.864	.026
Myo5a	.931	.007	Ccnf	-.697	.124
Paip2	.952	.003	Ccng1	.855	.030
Pgrmc1	.961	.002	Cdh1	.864	.027
Ptprd	-.815	.048	Colec12	.937	.006
Serpini1	.970	.001	Dhcr24	-.724	.104
Syap1	.990	.000	Dusp6	.949	.004
Vim	.999	.000	Eif1a	.899	.015
			Fam101b	.805	.053
			Grn	-.897	.015
			Gucy1b3	.883	.020
			Id2	.822	.045
			Igf2	-.714	.111
			Itgb3bp	.829	.041
			Itm2c	-.817	.047
			Maea	-.926	.008
			Myh9	-.892	.017
			Optn	.805	.053
			Padi2	.908	.012
			Pgrmc1	.878	.021
			Pqlc3	.940	.005
			Prnp	.952	.003
			Rasa1	.898	.015
			Smarcd2	-.916	.010
			Syap1	.921	.009
			Sypl1	.895	.016
			Tagln	.756	.082
			Vim	.859	.028
			Vps37a	.937	.006

We did not assess the mRNA expression changes in the prefrontal cortex of the adult females on P133. However, we performed correlation analysis to verify whether stress-induced mRNA expression changes in miRNAs targets in placenta and embryonic cortex correlate with miRNAs expression in P133 prefrontal cortex. We correlated miRNA expression changes in the prefrontal cortex of adult females on P133 with mRNA expression changes of validated miRNA targets in placenta and E21 cortex in the SNNN group.

Out of 13 targets of miR-92b-3p only one target, *Dlst*, was found to be significantly altered in the placenta of the SNNN group. No targets of miR-92b-3p were found to be altered in E21 cortex. The analysis showed negative correlation between miR-92b-3p expression in P133 SNNN cortex and *Dlst* mRNA expression in SNNN placenta (Pearson correlation coefficient = -0.861, p=0.027).

Similarly, we performed the correlation analysis of miRNA expression changes in prefrontal cortex of adult females on P133 (SSSS) and mRNA expression changes of miRNA targets (Table S10) in placenta and E21 cortex of F3 generation offspring (SSSN) in the cumulative stress exposure group (Tables 3.23-3.28).

Table 3.23. Results of correlation analysis of miR-30a-5p expression in prefrontal cortex of adult females on P133 (SSSS) vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 female offspring (SSSN).

miR-30a-5p					
mRNA	**Pearson Correlation**	**Sig. (2-tailed)**	**mRNA**	**Pearson Correlation**	**Sig. (2-tailed)**
Cortex			**Placenta**		
Acsl4	-.917	.010	Acsl4	-.904	.013
Atox1	.878	.021	Anxa1	-.840	.036
Atp6v1c1	-.897	.015	Atox1	.846	.034
Becn1	-.814	.049	Atp6v1b2	.883	.020
Cdc20	.949	.004	Atp6v1c1	-.876	.022
Ctnnb1	.817	.047	Becn1	-.772	.072
Dnajc2	.889	.018	Cbfb	-.819	.046
Faf2	.972	.001	Fahd1	-.919	.010
Kpna1	.913	.011	Igf1r	.783	.066
Lrrn1	-.945	.004	Man2b1	.725	.103

Mak16	.912	.011	Nde1	-.924	.009
Man2b1	.916	.010	Nip7	-.873	.023
Mpdu1	.727	.102	Pcna	-.831	.041
Ncl	.958	.003	Pdcd10	-.842	.036
Nol9	.896	.016	Pgm1	.944	.005
Pabpc1	-.890	.017	Ppid	-.880	.021
Pcna	-.908	.012	Ppp1r10	.726	.102
Pdcd10	-.908	.012	Ppp3r1	.900	.015
Pgm1	.887	.018	Retsat	.732	.098
Ppid	-.900	.014	Rpn2	.752	.085
Rab14	.920	.009	Stub1	.836	.038
Scaf1	-.866	.026	Trio	.926	.008
Sec11c	-.843	.035	Ubl3	-.908	.012
Tp53	.806	.053	Vim	-.921	.009
Tpi1	.911	.012	Ywhae	.822	.045
Ubl3	-.953	.003			
Vim	-.870	.024			

Table 3.24. Results of correlation analysis of miR-136-5p expression in prefrontal cortex of adult females on P133 (SSSS) vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 female offspring (SSSN).

miR-136-5p					
mRNA	**Pearson Correlation**	**Sig. (2-tailed)**	**mRNA**	**Pearson Correlation**	**Sig. (2-tailed)**
Cortex			**Placenta**		
Ccdc127	-.897	.015	Ccdc127	-.875	.022
Celsr3	.736	.095	Mgst1	-.768	.075
Gnaq	.842	.036	Tmem168	-.782	.066
Hmgcr	-.793	.060	Usp15	-.654	.159
Igfbp2	.835	.038	Xrcc6	.692	.128
Map2	-.641	.170			
Myt1l	-.920	.009			
Negr1	-.579	.228			
Nfib	-.711	.113			
Odf2	.740	.093			
Ppp3cb	.691	.128			
Slc12a5	-.833	.039			
Wdr70	.817	.047			
Zfp354c	-.708	.116			

Table 3.25. Results of correlation analysis of miR-30e-5p expression in prefrontal cortex of adult females on P133 (SSSS) vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 female offspring (SSSN).

miR-30e-5p					
mRNA	**Pearson Correlation**	**Sig. (2-tailed)**	**mRNA**	**Pearson Correlation**	**Sig. (2-tailed)**
Cortex			**Placenta**		
B4galt6	-.862	.027	Arid4b	-.939	.005
Becn1	-.735	.096	B4galt6	-.822	.045
Camk2n1	-.746	.089	Becn1	-.695	.125
Canx	.831	.040	Btbd9	.808	.052
Dio2	.817	.047	Canx	.584	.224
Enc1	.775	.070	Dap	.863	.027
Gpm6a	-.860	.028	Egln2	.870	.024
Gria2	-.967	.002	Hsp90aa1	-.808	.052
Hsp90aa1	-.845	.034	Igf1r	.849	.032
Lancl1	-.752	.084	Insig2	-.928	.008
Mcf2l	.820	.046	Myh9	.856	.030
Mcl1	-.890	.018	Pigk	.727	.102
Nfib	-.813	.049	Ppp3r1	.908	.012
Nid1	-.593	.215	Psma6	-.799	.056
Nsg1	.834	.039	Rps7	-.871	.024
Ptprd	.840	.036	Samd8	-.881	.020
Rps7	-.885	.019	Slc7a1	-.844	.035
Scn2a1	-.869	.025	Sv2b	-.681	.136
Slc6a15	-.879	.021	Tspyl1	-.881	.021
Slc7a1	-.830	.041	Ube2i	-.735	.096
Tp53	.784	.065	Ywhag	-.786	.064
Tspyl1	-.817	.047	Zfp36l1	-.906	.013
Ube2i	-.895	.016			
Ywhag	-.771	.073			
Zfp36l1	-.890	.018			

Table 3.26. Results of correlation analysis of miR-92a-3p expression in prefrontal cortex of adult females on P133 (SSSS) vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 female offspring (SSSN).

miR-92a-3p					
mRNA	**Pearson Correlation**	**Sig. (2-tailed)**	**mRNA**	**Pearson Correlation**	**Sig. (2-tailed)**
Cortex			**Placenta**		
Adrm1	.811	.050	Arcn1	-.848	.033
Cdc20	-.841	.036	Arid4b	.822	.045
Chn1	-.806	.053	Cbfb	.799	.056
Crbn	-.878	.022	Cdh1	.833	.040
Ddx56	-.872	.023	Ddx6	.924	.008
Ddx6	.836	.038	Dhcr24	-.721	.106
Dnm2	-.937	.006	Eftud2	-.963	.002
Etfa	.823	.044	Egln2	-.872	.023
Efnb1	-.904	.013	Etfa	.871	.024
Fkbpl	-.864	.027	Gak	-.985	.000
Gak	-.974	.001	Ezr	-.718	.108
Gapdh	-.894	.016	Gemin6	.826	.043
Gemin6	.676	.140	Ghitm	.728	.101
Gstp1	-.856	.029	Hnrnpf	.802	.055
Hbb	-.856	.030	Igf1r	-.940	.005
Hmgcr	.896	.016	Ik	.808	.052
Hsd17b10	-.872	.023	Kdelr1	-.974	.001
Ik	.860	.028	Mfn2	-.847	.033
Kdelr1	-.878	.021	Myh9	-.786	.064
Klhl36	-.960	.002	Optn	.713	.112
Mfn2	-.935	.006	Pdlim1	-.825	.043
Mlx	-.779	.068	Pfdn6	-.903	.014
Myef2	-.869	.025	Ppib	-.958	.003
Ncl	-.827	.042	Ppp4c	-.924	.008
Nfib	.913	.011	Prelid1	-.771	.073
Ogt	-.934	.006	Psmd2	-.974	.001
Optn	.848	.033	Rab11a	.808	.052
Pfkm	-.861	.028	Rangap1	-.883	.020
Plcg1	-.960	.002	Rasa1	.859	.028
Ppil1	-.907	.012	Rbbp7	.904	.013
Ppp2r1a	-.875	.022	Recql	.711	.113
Ppp4c	-.888	.018	Rnps1	-.971	.001
Psmd2	-.856	.030	Rpl18a	.908	.012

Psmb6	-.637	.174	Rpl24	.811	.050
Psmc3	-.817	.047	Rpl8	-.952	.003
Rab11a	.738	.094	Rplp1	-.692	.127
Rangap1	-.936	.006	Rps15	-.958	.003
Rasa1	.894	.016	Rps3a	.847	.033
Rbbp7	.874	.023	Safb	-.957	.003
Raver1	.845	.034	Scarb2	-.836	.038
Rbm10	-.902	.014	Serinc1	.918	.010
Riok1	-.755	.083	Slc7a1	.844	.035
Rpl18a	.912	.011	Smad4	.769	.074
Rps15	-.768	.075	Smarcd2	-.949	.004
Rps3a	.930	.007	Snf8	-.984	.000
Rxrb	-.849	.033	Stat3	-.842	.036
Serinc1	.738	.094	Surf6	-.873	.023
Slc12a5	.808	.052	Vim	.754	.084
Slc20a1	-.753	.084	Wee1	.796	.058
Slc3a2	-.925	.008	Ywhaq	.867	.025
Slc6a8	-.907	.013	Znf622	-.861	.028
Slc7a1	.877	.022			
Smad4	.854	.030			
Snf8	-.877	.022			
Tle3	-.910	.012			
Tmed9	-.898	.015			
Trappc2	.873	.023			
Ttc5	-.847	.033			
Vim	.919	.009			
Ywhaq	.835	.038			

Table 3.27. Results of correlation analysis of miR-128-3p expression in prefrontal cortex of adult females on P133 (SSSS) vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 female offspring (SSSN).

miR-128-3p					
mRNA	**Pearson Correlation**	**Sig. (2-tailed)**	**mRNA**	**Pearson Correlation**	**Sig. (2-tailed)**
Cortex			**Placenta**		
Atp6v1c1	.871	.024	A2m	.871	.024
Bpgm	-.863	.027	Atp6v1c1	.849	.033
Cdkn1b	.849	.033	Cdkn1b	.930	.007
Gnaq	-.944	.005	Dbi	.795	.059
Map2k1	.787	.063	Eif2s2	.045	.932

Pdpk1	-.929	.007	Fkbp10	.835	.038
Slc39a7	-.917	.010	Gltscr2	-.878	.021
Snap25	.917	.010	Hmox1	.931	.007
Tmem30a	.830	.041	Itpr1	.839	.037
			Klf4	-.894	.016
			Litaf	.787	.063
			Map2k1	-.929	.007
			Pdpk1	-.586	.221
			Ptgs2	.780	.067
			Sgpp1	-.917	.010
			Slc39a7	-.821	.045
			Tagln	.830	.041
			Tmem30a	.704	.119
			Tusc3	.661	.153
			Wee1	-.756	.082
			Xrcc6	-.738	.094

Table 3.28. Results of correlation analysis of miR-92b-3p and miR-101a-3p expression in prefrontal cortex of adult females on P133 (SSSS) vs. mRNA expression of validated miRNA targets in placenta and E21 cortex of F3 female offspring (SSSN).

Placenta		
miR-92b-3p		
mRNA	Pearson Correlation	Sig. (2-tailed)
Ddt	-.675	.141
Dlst	-.817	.047
Nde1	.792	.061
miR-101a-3p		
Akt1	.821	.045
Ptgs2	.131	.805

Discussion

The present study investigated miRNA expression changes in placenta and embryonic cortex of the offspring in three generations of animals. To identify if miRNA signatures of placenta may serve as a proxy for central changes in the brain with potentially predictive value, a special emphasis of this study was the comparison of cortical and placental miRNA expression profiles. Our results demonstrated that overall expression of 17 miRNAs was altered in response to stress in placenta and in the cortex of the offspring. Interestingly, the offspring's cortex was affected only in the F3 generation, where four miRNAs were altered in SNNN group (miR-331, -409-5p, -100 and -99b) and two miRNAs in SSSN group (miR-331 and -409-5p). Notably, the expression of the same cortical miRNAs was also found to be altered in placenta in the F3 generation, however, there were other miRNA expression changes in placenta that do not have a documented consequence on brain development (SNNN – 7 altered miRNAs; SSSN - 11). In addition to the changes in the F3 generation, six miRNAs were altered in the SSN group in placenta in the F2 generation. Interestingly, expression of miR-331 and -409-5p was altered in the cumulative stress exposure stress line in both F2 (SSN) and F3 (SSSN) generations in placenta, with similar expression pattern in SSSN group in the embryonic cortex in F3 offspring (Fig. 3.3). To verify whether observed miRNA expression changes in the embryonic cortex are long-lasting and remain into adulthood, we analysed prefrontal cortex of the F3 offspring on postnatal day P133. Overall, expression of 11 miRNAs was altered in the brain of adult F3 offspring, however, no overlaps between expression patterns in embryonic, or placenta and adult brains were found. Possible miRNA functions based on the data from the current literature are summarized in Tables 3.29 and S6.

Table 3.29. Summary of miRNA functions.

miRNA	Direction of miRNA change and/or miRNA function	Reference (PMID)
miR-331	Was up-regulated in n p53-/- Day 8.5 mouse embryos.	19229884
	Was decreased in embryonic homozygous Hmga1-knockout mouse.	19169275

	Was up-regulated in chronic and acute lymphocytic leukemias.	17934639
	Role in cell cycle-related biological processes.	19517021
	Was up-regulated in rat hepatocarcinogenesis.	21035526
	Was up-regulated after valproic acid (HDAC inhibitor) following by MCAO in rat (ischemia).	22937209
	Role cell cycle regulation.	19996289
	Was among expressed embryonic E18 rat neuronal miRNAs.	14691248
miR-100	Was among expressed embryonic E18 rat neuronal miRNAs.	14691248
	Was down-regulated in breast cancer cells, leads to an up-regulation of the proliferation- and survival-promoting oncogene insulin-like growth factor 2.	22926517
	Up-regulation is associated with poor survival in lung cancer.	22937028
	Was up-regulated in plasma of patients with endometrial carcinoma, down-regulated in endometrium; decreased expression was associated with shorter survival time.	22920721
	Was decreased in endometrioid endometrial carcinoma	21897839
	Was down-regulated in the semen of infertile males.	22735917
	Role in ovarian cancer.	22246341
	Was down-regulated in chronic obstructive pulmonary disease.	22686440
	Role in cell fate determination during nervous system differentiation.	22578317
	Role in DNA damage repair.	22525276
	Was up-regulated in endometriosis.	19074548
	Overexpression of miR-100 inhibited osteogenic differentiation of human adipose-derived mesenchymal stem cells *in vitro*, whereas down-regulation of miR-100 enhanced the process.	22684006
miR-370	Overexpression of miR-370 and down-regulation of its target TGFβ-RII contributed to the progression of gastric carcinoma.	21666718
	Was down-regulated in bladder cancer.	21304530
	Role in regulating cholesterol homeostasis, fatty acid metabolism and lipogenesis.	21178770
	Was up-regulated in endometrial carcinosarcoma.	21125666
	Was decreased in neuroblastoma cells in comparisons to mature neurons and neural progenitor cells.	22244746
	Relatively high expression of a cluster of three microRNAs including miR-134, miR-198, and miR-370 in unstable angina pectoris patients allowed to discriminate them from stable patients (potential biomarkers of risk for acute coronary syndromes).	20230787
	Controlled the expression of microRNA-122 and Cpt1alpha and affected lipid metabolism.	20124555

	Together with other miRNAs positively correlated with the severity of ischemic injury.	19780683
miR-153	Together with miR-16 and miR-590-5p was used in rectal cancer as predictors of response to neoadjuvant chemoradiation therapy.	22903298
	Role in apoptosis, proliferation in glioblastoma.	22893786
	Inhibited expression of amyloid-β precursor protein in human neurons by specifically interacting with the APP 3'-UTR.	22733824
	Was up-regulated after treatment with temozolomide and *Olea europaea* leaf extract in human glioblastoma cells.	22722712
	Negatively regulated the expression of amyloid precursor protein and amyloid precursor-like protein 2. Was decreased at early- and late-stage of AD in APPswe/PSΔE9 murine model.	22510281
	Expression was altered in fetal cerebral cortical-derived neural progenitor cells after the exposure with ethanol and nicotine.	22458409
	Role in Parkinson disease. Together with mir-7 post-transcriptionally regulated alpha-synuclein expression.	20106983
	Role in Alzheimer's disease. Might regulate APP expression *in vitro* and under physiological conditions in cell.	21982160
	Cytosine methylation in miR-153 gene promoters increased the expression of holocarboxylase synthetase, thereby increasing the abundance of histone H4 biotinylation marks in HEK-293 human kidney cells.	21764280
	Was down-regulated in As2O3-induced drug-resistant K562 cells in the CD34+ K562 subpopulation, which is characteristic of leukemia stem cell and resembles the drug-resistant subgroup.	21267675
	Was up-regulated in bleomycin-induced pulmonary fibrosis.	21266501
	Down-regulation of miR-153 and up-regulation of miR-519a were correlated significantly with advanced clinical stage in ovarian epithelial tumours.	21083603
	Is a tumor suppressor, inhibited the protein kinase B pathway via reducing the protein level of insulin receptor substrate-2.	21213215
	Expression of miR-9, miR-27, miR-96, miR-153, miR-182, miR-183, or miR-186 was sufficient to significantly reduce the abundance of FOXO1 in endometrial cancer.	20028871
	Was enriched in the brain.	22775435
	Is a brain-specific miRNA that was expressed at a significantly lower level in glioblastoma relative to non-neoplastic brain tissue, down-regulated both Bcl-2 and Mcl-1.	19676043
miR-181a	Role in apoptosis, colony formation, invasion, proliferation, radiosensitivity in glioblastoma.	22893786
	Was down-regulated in acute promyelocytic leukemia cells during differentiation.	22967415
	Was up-regulated in the bovine mammary gland during lactation.	22959945

	Was down-regulated in mastitis and associated with regulation of innate immunity and mammary epithelial cell function in tissue challenged with *Streptococcus uberis*.	22959936
	Repressed ox-LDL-stimulated inflammation response in dendritic cell by targeting c-Fos.	22956783
	Role in osteoblastic differentiation of mouse induced pluripotent stem (iPS) cells.	22937097
	Controls the development of normal thymic T cells and leukemia cells.	22916024
	Targeted 3'-UTR of PRKCD gene; expression level of miR-181a in cervical cancer may serve as a biomarker for sensitivity to radiation therapy.	22847611
	Was up-regulated in children with newly diagnosed Type 1 Diabetes.	22829805
	May bind to TGFBR1 and TGFBRAP1 3'-UTRs.	22714950
	May represent a novel biomarker for primary breast cancer, was down-regulated in the serum of breast cancer patients.	22692639
	Was up-regulated in gastric cancer.	22971574
	Suggested role in differentiation of human embryonic stem cells to vascular endothelial cells.	22232059
	Was down-regulated in preterm birth.	21079238
	Was up-regulated in multiple myeloma.	22447484
miR-99b	Was among expressed embryonic E18 rat neuronal miRNAs.	14691248
	miR-99b acted as a tumor suppressor in non-small cell lung cancer by directly targeting fibroblast growth factor receptor 3.	22969861
	Was down-regulated in spleen and liver in mice with *Plasmodium chabaudi* (malaria).	22562236
	Was up-regulated in multiple myeloma.	22447484
	Was co-expressed in atherosclerotic plaques and classical monocytes in humans.	22370758
	Modulated TGF-β induced epithelial to mesenchymal plasticity in normal murine mammary gland cells.	22299047
	Together with miR-181a, and -181b comprised a component of an endothelial-miRNA signature; may enhance differentiation of human embryonic stem cells to vascular endothelial cells.	22232059
	May contribute to the development castration-resistant prostate cancer.	22266859
	Plays a role in regulation of TRAF2 and NFκB pathway in breast cancer.	22167321
	Plays a role in regulation of primitive hematopoietic cells.	22123844
	MiR-99b, miR-212, and miR-511 were detected as the three most highly up-regulated miRNAs both in dendritic cells and macrophages.	21646346
	Was up-regulated in endometriosis.	19074548

	Role in intestinal epithelial cell fate.	20638171
	Role in melanoma.	20302635
	Role in multiple myeloma.	19846888
	Was down-regulated in adenocarcinoma tissues.	19077565
	Was up-regulated in synovial sarcoma.	21213367
miR-9	Was decreased expression in neuroblastoma cells in comparisons to mature neurons and neural progenitor cells.	22244746
	Was increased in endometrial carcinoma.	21897839
	Role in glioblastoma.	22122801
	Role in glioblastoma.	22893786
	Role in brain development, neurogenesis, spinal motor neuron development, malfunction in spinal motor neuron disease, coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors, regulated REST in HD, brain primary tumors (review).	21697652
	Role in fetal alcohol syndrome; was up-regulated in brain after prenatal ethanol exposure.	19091803
	Was altered expression in fetal cerebral cortical-derived neural progenitor cells after ethanol and nicotine exposure.	22458409
miR-29a	Was decreased in homozygous Hmga1-knockout mouse embryonic fibroblasts MEFs in comparison with wild-type cells.	19169275
	Was up-regulated in children with newly diagnosed Type 1 Diabetes.	22829805
	Was among expressed embryonic E18 rat neuronal miRNAs.	14691248
	Was up-regulated in chronic and acute lymphocytic leukemia.	17934639
miR-30e*	Was up-regulated in p53-/- Day 8.5 Embryos mouse.	19229884
	Role in ovarian cancer.	24952258, 24676806
	Role in non-small cell lung cancer.	24945821
	Was up-regulated in high-fat diet-fed fish.	24788396
	Role in in gastrointestinal cancer, potential regulator of Bmi1 expression.	24312366
	Signature in neutrophils after traumatic injury.	24108763
	Was enriched in myocardium.	23300973
	Was down-regulated in malignant peripheral nerve sheath tumours compared to neurofibromas.	
	Role in different set of gliomas.	23006329
	Potential biomarkers for smoking-induced lung disease.	22512273
	Was up-regulated in primary human glioma cells and correlated with malignant progression and poor survival. Potential therapeutic target and prognostic marker.	22156201

	Role in colorectal cancers.	21963845
	Role in long-term high-fat diet-induced obesity in mice.	22496873
	Role in lung cancer. Correlated with pathologic parameters and survival of patients with lung cancer.	20595154
	Role in melanoma.	20302635
miR-154	Role in neuroplasticity, axonal guidance, domaminergic neuron differentiation morphine self-administration behavior.	22804800
	Role in medullary thyroid carcinoma.	22747440
	Was inversely correlated with those of CCND2 in hepatocellular carcinoma.	21128228
	Role in cis-regulatory modules during embryonic development.	21059252
	Role in primary acute myeloid leukaemia.	20827525
	Role in fetal alcohol syndrome. Was down-regulated in brain after prenatal ethanol exposure.	19091803
	Role in acute myeloid leukaemia.	18478077
	Was significantly overexpressed (8.fold) in familial and sporadic medullary thyroid carcinoma.	22550943
	Role in Graves' disease.	22456620
	Role in cis-regulatory modules in embryonic development.	21059252
	Role in erythropoiesis.	17379065
	Appeared to be localized to the stroma of fetal but not adult lungs.	17191223
miR-675*	Was decreased in early-onset preeclamptic placentas.	22832245
	Was shown to be expressed exclusively in the placenta from the gestational time point when placental growth normally ceases. Overexpression of miR-675 in a range of embryonic and extraembryonic cell lines resulted in their reduced proliferation. Was shown to target *Igf1r in vitro*.	22684254
	Was shown to be significantly increased in ischemia.	24574964
miR-493*	Organ-specific testosterone-insensitive response of miRNA expression of C57BL/6 mice *to Plasmodium chabaudi* malaria. In the liver, miR-376B, miR-493*, and miR-188-3P were upregulated by 2.4-fold, 2.2-fold, and 2.1-fold, respectively.	22562236
	miR-130a, miR-199b-3p, miR-200b, and miR-125b were down-regulated, whereas miR-342-3p, miR-432, miR-23b, miR-493, miR-493*, and miR-664* were up-regulated prolactinoma.	22490835
	miR-493 induction during carcinogenesis blocked metastatic settlement of colon cancer cells in liver. Functional analyses showed that miR-493 and to a lesser extent miR-493* were capable of inhibiting liver metastasis.	22373578
	miR-127-3p, miR-184, miR-195 and miR-493∗ were enriched in the pancreatic islets of glucose intolerant human subjects in comparison to healthy controls.	21094635

	Expression profile of microRNAs and mRNAs in human placentas from pregnancies complicated by preeclampsia and preterm labor. miR-181a was down-regulated in preterm birth miR493 was upregulated.	21079238
	Cancer-specific alterations of microRNA expression in human male breast cancer. miR-493-5p was identified as most prominently up-regulated.	20331864
	miRNA profiles of malignantly transformed human bronchial epithelial cells. The lowest expressed miRNAs were miR-10a, miR-493-5p, and miR-363*.	19462682
	LongSAGE data suggested the existence of a mouse homolog of human and rat mir-493. Analysis of 29 human and 230 mouse longSAGE libraries revealed the expression of 22 known and 10 predicted mammalian miRNAs. Most were detected in embryonic tissues.	17090314
	Was among expressed embryonic E18 rat neuronal miRNAs.	14691248
miR-322*	Up-regulated expression in serum and amygdala in a rat model of posttraumatic stress disorder (PTSD). Potential biomarker of PTSD.	24998397
	Was induced in response to hypoxia.	18316553
	Was specifically enriched in oligodendrocytes.	23516279

Stress exposure of great-grandmother during gestation may potentially alter important molecular pathways in the brain of great-grand offspring

The main purpose of this research was to investigate the mechanisms of transgenerational transmission of stress responsiveness in a rat model. We hypothesized that prenatal stress causes heritable changes in the brain of the offspring that persist through generations. We also hypothesized that prenatal stress causes miRNA expression changes in placenta that contribute to the subsequent genetic and epigenetic changes in the brain of the fetus. MiR-99b and miR-100 were found to be altered in both placenta and embryonic cortex in the SNNN group, suggesting that changes in the placenta may contribute to the changes in miRNA expression in the cortex. MiRNAs that were altered in response to stress in parental generation have multiple targets. The analysis of mRNA targets using DIANA-miRPath showed that targets of the altered miRNAs may be involved in important neurological pathways, including axon guidance, long-term depression, Wnt- and TGF-beta signaling pathways. Heritable transgenerational changes in miRNA

expression in fetal cortex and placenta, as well as miRNA functions and cumulative effects of stress will be discussed below.

Heritable transgenerational changes in miRNA expression in fetal cortex and placenta

The main focus of this research was to identify miRNAs that show possible transgenerational inheritance, therefore, changes in F3 generation in the non-cumulative stress exposure lineage (SNNN) are of most interest. Four miRNAs were found to be altered in the embryonic cortex in the SNNN group with similar expression patterns in placenta, including miR-100 and miR-99b that are broadly conserved across most vertebrates and were up-regulated in response to stress. Interestingly, fibroblast growth factor receptor 3 (FGFR3) is a common target of both miRNAs. Our Western immunoblotting results showed that FGFR3 protein was significantly down-regulated in cortex in SSSN group and in placenta in SNNN group, suggesting that these two miRNAs may be involved in transgenerational regulation of FGFR3 expression in placenta and the brain. FGFR3 plays an important role in brain development (Inglis-Broadgate et al 2005, Moldrich et al 2011, Oh et al 2003). For example, it was demonstrated in the mouse model that FGFR3 controls development of the cortex (Inglis-Broadgate et al 2005). Mutation in this receptor resulted in the enlargement of the embryonic brain in mice, possibly via regulation of progenitor cell proliferation and apoptosis (Inglis-Broadgate et al 2005). Accordingly, Moldrich et al. (2011) showed that brain weight and overall size, as well as cortical and hippocampal volumes were reduced in *Fgfr3* knockout mice (Moldrich et al 2011). Other possible functions of miR-99b and miR-100 will be discussed in more detail below.

Possible function of miR-99b in pathological conditions

MiR-99b was shown to be highly expressed in the brain (Kane et al 2012). The level of miR-99b was found to be significantly lower in the cerebrospinal fluid of patients with fibromyalgia (FM) in comparison to healthy controls (Bjersing et al 2013). FM is a disease characterized by chronic pain and reduced pain threshold. The pathophysiology of FM involves impaired functioning of neuroendocrine system, including altered growth hormone/insulin-like

growth factor-1 axis (Bjersing et al 2013). Interestingly, we observed the down-regulation of insulin-like growth factor 1 receptor in both placenta and embryonic cortex in the offspring of F3 generation. Altered expression of miR-99b was also implicated in the reproductive system (Resnick et al 2009). Resnick at al. (2009) found decreased levels of circulating miR-99b in the serum in patients with ovarian cancer.

Possible functions of miR-100 in stress response and aging

MiR-100 is generally implicated in the brain stress response. For example, Haramati et al. (2011) showed that miR-100 was up-regulated in amygdala 90 minutes after acute stress (Haramati et al 2011). Over-expression of miR-100 was shown to down-regulate ATM in a human glioma cell line (Ng et al 2010). MiR-100 was also shown to be up-regulated during aging in the mouse brain (Li et al 2011). Interestingly, miR-100 was up-regulated in the third trimester placentas compared to the first trimester placentas in humans (Gu et al 2013). Insulin-like growth factor 1 receptor (IGFR1) is among the targets of miR-100 and our results of Western immunoblotting showed down-regulation of this protein in the placenta and embryonic cortex of F3 offspring. Expression levels of miR-100 in embryonic cortex in the F3 offspring in the SNNN group also correlated with the *Igfr1* mRNA expression in placenta in the SNNN group.

Cumulative effects of stress in multiple generations on miRNA expression in placenta

Our results showed significant changes in the expression of six miRNAs (miR-409, -9, -29a, -493*, -3596d, -331) in placenta in the F2 generation of the cumulative stress exposure lineage (SSN). Furthermore, there were seven miRNAs (miR-99b, -322*, -30e*, -331, -409, -154, -871*) altered in the F3 generation of this lineage (SSSN). MiR-331 and miR-409 were common between the two generations in the cumulative stress exposure lineage and were also altered in the embryonic cortex in the offspring of F3 generation. Thus, at least for these two miRNAs, expression patterns in placenta are predictive of the changes in the miRNA expression in embryonic cortex. Interestingly, altered expression of miR-331 at the maternal-fetal interface was implicated in spontaneous fetal loss during early pregnancy in a pig model (Wessels et al 2013).

The present results revealed down-regulation of miR-331 in placenta in response to stress. Wessels et al. (2013) also showed that miR-331 expression was decreased in endometrium associated with arresting concepti in comparison to endometrium associated with healthy concepti (Wessels et al 2013). Thus altogether these data suggest an important role of miR-331 in pregnancy outcomes. Overall our findings highlight the intriguing possibility that placental tissue can be used to predict alterations in the brain and they suggest that placental miRNAs can possibly be used as biomarkers of brain diseases.

Chapter 4: Transgenerational effects of gestational stress on DNA methylation

Introduction

DNA methylation plays a critical role during mammalian development (Li et al., 1992). It has a variety of functions and is required for several processes, such as inactivation of X-chromosomes, genomic imprinting and silencing of transposable elements and pericentromeric repeats to ensure genome integrity (Feil & Khosla 1999, Kaneko-Ishino & Ishino 2010, Sado et al 2004, Walsh et al 1998). DNA methylation has been shown to play an important role in perinatal programming that affects functioning of the hypothalamic-pituitary-adrenal axis and stress responses (Meaney & Szyf 2005, Meaney et al 2007, Mueller & Bale 2008). A number of studies have shown transgenerational changes in the DNA methylation of genes in response to prenatal and early-life stress (Dias & Ressler 2014, Franklin & Mansuy 2010, Franklin et al 2010, Weiss et al 2011). Our previous experiment (Chapter 2) showed that prenatal stress caused significant changes in the mRNA expression in placenta and embryonic brain in the offspring of F2 and F3 generations of prenatally stressed animals, suggesting transgenerational epigenetic inheritance. DNA methylation is known to play a role in mediating gene expression (Suzuki & Bird 2008), therefore we hypothesised that observed changes in the gene expression in response to stress could be regulated by the altered levels of DNA methylation at gene promoters.

Materials and methods

Animals

For the description see Animals section in Chapter 2.

Experimental groups

For the experimental design see Experimental groups section in Chapter 2.

Time course

For the time course description see Time course in Chapter 2.

Stress paradigm

The stress paradigm is described in Chapter 2.

Brain tissue dissection and RNA extraction

For the details on tissue dissection and RNA extraction see Chapter 2.

DNA methylation analysis

DNA methylation analysis of placental tissues and embryonic cortex was performed using reduced representation bisulfite sequencing, as described below.

Library preparation and sequencing

Genomic DNA (2 mg) extracted from rat cortex and placenta was digested with restriction enzyme *MspI* (Fermentas) that restrict CCGG site for 18 h at 37 C°. The fragments were blunt-ended and phosphorylated, a single A nucleotide was added to the 3' ends using Klenow enzyme and T4 DNA polymerase (Fermentas). The fragments were ligated to methylated adapters, resolved on 2% gel prepared with Low Range Ultra Agarose and size selected to include those of 200 to 300 nucleotides (nt) in length. Ligated fragments purified from the gel, using QIAquick gel extraction kit (QIAGEN), were subjected to bisulfite conversion using EZ DNA Methylation-Gold kit (ZymoResearch). Bisulfite converted libraries were purified and sequenced on an Illumina GAIIx genome analyzer using single-end 36 cycle protocol, 6 samples were multiplexed per each flow cell lane.

Base calling, demultiplexing and initial quality control

Base calling and demultiplexing was performed using Illumina CASAVA 1.8.2 with default settings following the developer's manual (Illumina). Resulting sequencing reads were obtained in fastq format with base qualities in Sanger format (Phred + 33). Initial quality control (QC) was performed using NGSQCtoolkit version 2.3 (Patel and Jain, 2012) and involved examination of average read qualities, read quality distributions, read base composition and GC content.

Read trimming and mapping to the reference genome

Sequencing reads were trimmed of adapters, low quality ends with the quality cut-off of 25 and 2 base pairs (bp) from the 3' end to avoid the use of filled in cytosine position for methylation calls. The trimming was performed with Trim Galore! v. 0.2.7 software (Babraham Bioinformatics).

Data analysis and statistics

Detection of differentially methylated regions (DMRs)

Alignment files in Sequence Alignment/Map (SAM) format output by Bismark were loaded into R Version 3.0.1 (R Development Core Team) using functions implemented in methylKit Bioconductor package (Akalin et al 2012). MethyKit functions were used to build histograms of reads coverage and distributions of CpG methylation for every sample library as a mean to provide additional quality information.

Detection of DMRs was performed using BiSeq version 2.14.1 Bioconductor package (Hebestreit et al 2013). DMR detection algorithm implemented in BiSeq was designed to analyze low coverage data typical in RRBS and takes into account high correlation in methylation status between closely located CpGs (Eckhardt et al 2006). The DMR detection algorithm was described in detail by Hebestreit et al. (2013) and the analysis was performed according to BiSeq user manual available at http://bioconductor.org/packages/release/bioc/html/BiSeq.html (Hebestreit et al 2013). Briefly, text files in bedgraph format containing coverage and per CpG methylation information were loaded into R using data input function implemented in BiSeq. First, we identified frequently covered CpG sites as those that are covered in at least 75% (4/5) samples. Frequently covered CpGs located no further than 100 base pairs (bps) were merged into CpG clusters, each cluster was set to contain at least 5 frequently covered CpG sites. CpGs with extremely high coverage (> 99%) were excluded from the analysis.

A local smoothing procedure was applied to predict methylation status of CpGs with low coverage within clusters. Previously, it was shown that smoothing decreases sequencing coverage

requirements and prevents data loss caused by exclusion of low coverage CpGs (Hansen et al 2012). The smoothing procedure implemented in BiSeq ensured that high coverage CpGs had greater influence on estimation of methylation level within the CpG cluster. Next, beta regression was fitted to the smoothed methylation levels and group effect tests were performed using the Wald test. Multiple comparisons adjustment was performed according to hierarchical testing procedure suggested by Benjamini and Heller (2007), in which regions are tested first and then locations are tested within rejected regions, thus reducing the number of hypotheses tested and increasing statistical power (Benjamini & Heller 2007).

CpG clusters containing at least one differentially methylated CpG site were trimmed of non-significant CpG sites. Significantly changed CpG sites located no further than 100 bps were aggregated into DMRs. The DMR was divided, if methylation levels inside of it change in opposite directions, making sure that each DMR was either hyper- or hypo-methylated.

Annotation of differentially methylated regions

Genomic coordinates of DMRs detected by BiSeq were checked for overlaps with promoters and CpG islands using the ChIPpeakAnno Version 1.4.2 Bioconductor package, resulting in DMR intervals with associated p-values, methylation levels, presence of overlaps with promoter, CpG islands and gene descriptions was saved as final data. Coordinates of promoters were obtained by adding 2000 bps up- and down-stream of transcriptional start sites using the UCSC table browser (Karolchik et al 2004) and CpG island coordinates for rn4 Rat genome were obtained from the same source.

Results

To verify whether observed changes in the mRNA expression in response to prenatal stress were caused by altered levels of DNA methylation at promoter regions, we performed DNA methylation analysis of placental tissues and embryonic cortex in three generations of animals, using reduced representation bisulfite sequencing. Our results showed several significant changes

(>25% methylation difference, q-value<0.01) in the number of differentially methylated sites (varying from 1 to 54 in different group comparisons) in both placenta and embryonic cortex. DNA methylation analysis results are summarized in Table 4. Most of the changes were observed in the promoter regions of the genes (Table 4). For more details about the genes that were differentially methylated at the promoter region see Supplemental Materials (Tables S11, 12).

Table 4. Summary of DNA methylation analysis in placenta and embryonic cortex.

Generation	**Group comparison**	**Total # of regions with diff. methylation**		**# of regions with diff. methylation at promoters**	
		placenta	**cortex**	**placenta**	**cortex**
F1	SN vs. CT	31	3	22	1
F2	SNN vs. CT	9	15	3	12
	SSN vs. CT	54	7	40	5
	SNN vs. SSN	38	10	30	7
F3	SNNN vs. CT	24	5	16	5
	SSSN vs. CT	9	1	8	1
	SNNN vs. SSSN	10	8	9	3
F2 vs. F3	NNN vs. NNNN	41	8	29	3

In general, our results showed that the percentage of methylated CpGs was higher in embryonic cortex than in placenta (Fig. 4).

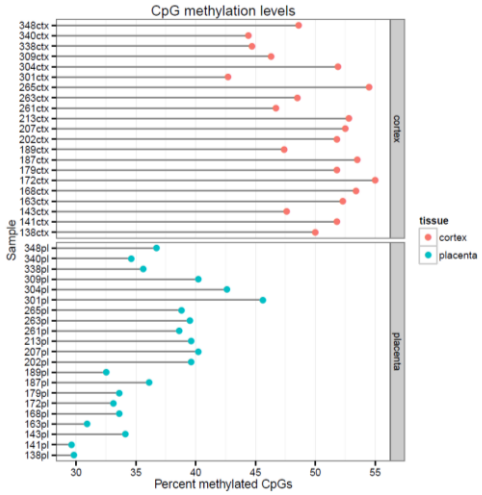


Figure 4. Total percentage of methylated CpGs in placenta (blue dots) and embryonic cortex (red dots). Samples: 138, 141, 143 (NNN); 163, 168, 172 (SN); 179, 187, 189 (SNN); 202, 207, 213 (SSN); 261, 263, 265 (NNNN); 301, 304, 309 (SNNN); 338, 340, 348 (SSSN).

DNA methylation changes vs. mRNA expression in the F3 generation

Placenta

Our results showed that 16 genes were differentially methylated at promoter regions in placenta. Out of these 15 genes were hypermethylated (including *Nrg1, Atoh8, Zpf704, Npr1, Fzd1, Trim33, Cyp2u1, Btbd1, Cdc42, Lrig3,* and *Slc25a10*), whereas only one gene was hypomethylated (*Amh*) in placenta in the SNNN group in comparison to the control group. Only one gene from the 16 genes with altered methylation levels at promoters was found to be altered on mRNA level in our mRNA profiling - *Atoh8*. Our mRNA profiling results showed that expression of *Atoh8* was decreased in SNNN group in comparison to controls (fold change (FC) = 0.7), whereas methylation of this gene at the promoter region was 73% higher in the same group.

When we compared changes in the single-generation stress exposure lineage with cumulative stress exposure (SNNN vs. SSSN), we found that nine genes were hypermethylated at promoter regions (including *Syn2, St8sia2, Mki67, Carm1, rno-miR-9b-1,* and *Phf12*). Our mRNA profiling results showed no changes in the expression of these genes. When animals from the cumulative stress exposure group (SSSN) were compared to their respective controls, eight genes were found to be differentially methylated at promoter regions, including *Actl6b, Bfsp1, Npw, Gnaq, Nup133,* and *Derl1*. There were no changes observed in the mRNA expression of corresponding genes.

Cortex

Our result showed that only one gene (*Deaf1*) was differentially methylated at the promoter region in the embryonic cortex in the SNNN group in comparison to control levels. *Deaf1* was also found to be altered on the mRNA level in this group. *Deaf1* had a 70% higher methylation level at the promoter region in the embryonic cortex of F3 offspring in the SNNN group when compared to the F3 offspring of control animals, whereas *Deaf1* mRNA expression was decreased (FC=0.75).

The expression of three genes (*Ajuba*, *Pde5a*, and *Ubn2*) that were differentially methylated at promoter regions in the SSSN vs. SNNN group was not altered in our mRNA profiling results. Only one gene (*Arhgef17*) was differentially methylated at the promoter region in the SSSN group in comparison to control with no observed changes at the mRNA expression level.

DNA methylation changes in F1 and F2 generations

Placenta

Our results of DNA methylation analysis in the F1 generation showed that 22 genes were differentially methylated at promoter regions in the SN group in comparison to the control group. One gene (*Sstr5*) was hypomethylated, whereas 21 genes were hypermethylated (Table S11). Three genes in the SNN group of the F2 generation in comparison to the control group had different methylation levels at promoter regions, including *Cd79a*. By contrast, 40 genes were differentially methylated at promoters in the cumulative stress exposure group (SSN). Thirty genes were found to be differentially methylated at promoter regions in the SSN vs. SNN comparison in placenta. One gene (*Slc9a2*) was hypomethylated at the promoter, whereas 29 genes were hypermethylated in SSN animals in comparison to the SNN group (Table S11). A comparison between controls in the F2 and F3 generations (NNN vs. NNNN) showed significant changes in the DNA methylation in 29 genes (27 hypomethylated, two – hypermethylated, including *Hck* and *Hes3*).

Cortex

Our results of the DNA methylation analysis in embryonic cortex revealed that only one gene (ENSRNOT00000050876) was differentially methylated in the SN group in comparison to controls. In the F2 generation (SNN vs. CT) 12 genes were differentially methylated at promoters, including *Zfp644, Camta2, Tbx18, Plcl2, Epc1, Ctgf, Snta1, Nr4a2,* and *Ndufb11*. Two genes out of 12 were found to be altered also on the mRNA level in our mRNA profiling results, *Ctgf* (FC=1.5) and *Snta1* (FC=0.75). Five genes showed significant differences in the methylation level

at promoter regions in the SSN group in comparison to control, including *Rbm18, Fdx1, Hspa9,* and *Tsen2*, however, no difference in the mRNA expression of these genes was observed. In the SSN vs. SNN comparison 7 genes were differentially methylated at promoters, including *Parp1, Dnaja1, Prmt3, Isyna1,* and *Sgms*. There were no changes in the expression of these genes in the mRNA profiling. Comparison between control animals in F2 and F3 generations (NNN vs. NNNN) revealed three genes with altered levels of DNA methylation at promoter regions, including *Kbtbd6, Ptma,* and *Polq*.

Discussion

Our mRNA profiling showed transgenerational changes in mRNA expression in placenta and embryonic cortex in response to prenatal stress in parental generation. To verify whether altered mRNA expression was regulated by changes in the levels of DNA methylation, we analysed DNA methylation changes in placentas and embryonic cortex in three generations of animals. Our results revealed a very small number of differentially methylated regions in the offspring of each generation in both tissues in response to stress. Our mRNA profiling showed almost no changes (0-2 genes) in placenta and embryonic cortex in the F1 generation, however, there were hundreds and thousands of genes altered in the offspring of subsequent F2 and F3 generations. In contrast, DNA methylation analysis of placenta and embryonic cortex revealed similar changes in each generation and only a small number of genes (1-54) showed significant changes at the level of DNA methylation. Comparison between control animals in the F2 and F3 generations also showed a similar range of changes. These results could indicate that observed changes in placenta and in the offspring of stressed animals were within background variation levels, or alternatively it can be due to the bystander effects of stress on control animals.

Interestingly, we did not observe any overlaps between the changes in DNA methylation in placenta and embryonic cortex, possibly indicating tissue specific changes at the level of DNA methylation in response to stress. Our findings revealed that the total percentage of methylated

CpGs in placenta was lower than in embryonic cortex. This is in accordance with the findings that the placenta has lower global DNA methylation profile than other tissues (Gama-Sosa et al 1983a, Grigoriu et al 2011, Perrin et al 2007).

There could be several explanations for the lack of correlation between mRNA expression and DNA methylation changes that were observed in the present study. First, promoter methylation is often highly dynamic during development. Thus, gene expression could be regulated by variable methylation outside CpG islands (Suzuki & Bird 2008), or by partially methylated domains that are present in developing tissues and placenta (Schroeder et al 2013, Schroeder et al 2011). It is also becoming increasingly evident that DNA methylation also occurs in non-CpG context (Lister et al 2013, Ramsahoye et al 2000, Tomizawa et al 2011, Ziller et al 2011), however, the role of non-CpG DNA methylation in the regulation of gene expression during development remains yet to be identified. Second, it is possible that immediate effects of stress in the F1 generation newborns occurred through other mechanisms of regulation, such as at the level of histone modifications and chromatin structure, or non-coding RNAs other than miRNAs. Such epigenetic changes were potentially inherited and became evident at the level of gene expression in subsequent F2 and F3 generations. Alternatively, there could be significant epigenetic changes that were not measured in the germ-line of F1 offspring or other developing organs that were not immediately reflected in the gene expression in the fetal brain, but became more evident later in adulthood and during pregnancy. Thus, these changes may have contributed to the passage of epigenetic information to the F2 and F3 offspring. Another possibility is that the sample size (n=3) used in this study was too low to produce reliable data. However, several studies that asses levels of DNA methylation in many generations of animals found significant changes using the sample size of three animals (Manikkam et al 2012, Skinner et al 2013b, Tracey et al 2013). The function of some of the genes with altered DNA methylation level will be discussed in more detail below.

Role of *Atoh8* and *Deaf1* in embryonic development

Atoh8

Our results demonstrated that *Atoh8* was decreased in the placenta of the SNNN group in comparison to control levels (fold change (FC) = 0.7), whereas DNA methylation of *Atoh8* at the promoter region was 73% higher in the same group. Thus, it is likely that reduced expression of *Atoh8* is due to hypermethylation of its promoter region. *Atoh8* is a transcription factor that is essential for the development of several tissues during embryogenesis (Guttsches et al 2015). *Atoh8* has been reported to play a role in the development of neurons, pancreas and kidney, retina and skeletal muscle (Inoue et al 2001, Lynn et al 2008, Ross et al 2006, Yao et al 2010).

Deaf1

Our results showed that *Deaf1* expression was decreased (FC=0.75) in the embryonic cortex of F3 offspring in the SNNN group when compared to control animals, whereas the level of DNA methylation at the promoter region of this gene was 70% higher. Thus, it is likely that the decrease in *Deaf1* expression in the cortex is regulated by altered DNA methylation levels in response to prenatal stress. *Deaf1* is a transcription factor that binds to its own promoter and *Hnrpa2b1* gene and down-regulates transcription of these genes (Huggenvik et al 1998). *Deaf1* plays an important role during embryonic development and is required for neural tube closure and skeletal patterning (Hahm et al 2004).

Conclusion

The present study investigated the role of DNA methylation in transgenerational stress response in rats. Surprisingly, we observed little effects of prenatal stress on DNA methylation patterns in either the brain or placenta. Changes in DNA methylation in placenta were shown previously to be associated with pregnancy complications, such as preterm birth, fetal growth restriction and preeclampsia (Banister et al 2011, Filiberto et al 2011, Kulkarni et al 2011). Interestingly, our previous experiments with the same stress paradigm showed that prenatal stress promoted preterm birth in the offspring of F2 and F3 generations (Yao et al 2014). These findings

suggest that epigenetic factors other than DNA methylation (e.g. histone modifications, non-coding mRNAs) may be involved in transgenerational inheritance of the stress-induced pathologies.

Chapter 5: General conclusions

The main goal of the present study was to investigate the mechanisms of transgenerational epigenetic inheritance of the stress response in rats. Several lines of evidence from both human epidemiological studies and research in animal models have indicated that stressful experiences during pregnancy and early-life could have long-term consequences for the future wellbeing of the mothers and their offspring (Talge et al 2007b). Animal and human research suggests that prenatal stress and stressful early-life experiences may increase the risks of neurological and psychiatric disorders (Franklin & Mansuy 2010, Huizink et al 2004). Notions from human epidemiological data highlighted the intriguing possibility that stressful events that were experienced by our ancestors during critical periods of development can influence subsequent generations, such as grandfathers' stressful experience can increase the risks of cardiovascular diseases and diabetes in grandchildren (Bygren et al 2001, Kaati et al 2002). These transgenerational effects may be regulated by epigenetic factors, such as miRNA expression, DNA methylation, and histone modifications that are prone to changes in response to stressful experiences and hostile environment. Animal models confirmed the possibility of stress-induced transgenerational epigenetic inheritance (Crews et al 2012, Dietz et al 2011, Franklin et al 2011, Gapp et al 2014, Ward et al 2013). Most of the studies on transgenerational epigenetic inheritance focused on the changes in the DNA methylation, whereas transgenerational changes in the miRNA expression are less frequently studied.

Our results demonstrated that ancestral exposure to stress during gestation caused significant changes in mRNA and miRNA expression as well as changes in DNA methylation in placenta and embryonic cortex in the offspring of the F3 generation, that have not been exposed to any direct or indirect stress. Thus, these findings indicate transgenerational inheritance of stress response in rats. Some of the F3 transgenerational responses were also observed in the F2 generation, however our results demonstrated that prenatal stress exposure had no immediate effect on the mRNA and miRNA expression in F1 offspring. Observed changes were similar in

the cumulative and non-cumulative stress exposure groups. Importantly, there was an overlap in mRNA and miRNA expression changes between placenta and cortex, suggesting that placenta has a potential to predict mRNA and miRNA changes in the brain. We found a correlation between miRNA expression changes and mRNA expression of confirmed miRNA targets in both placenta and embryonic cortex. Interestingly, some of the miRNAs did not reveal an inverse correlation to their targets on the mRNA level, likely because there are additional factors (other miRNAs) targeting these genes. Alternatively, there is a possibility that single miRNA targets many genes. Our mRNA and miRNA analysis revealed that stress altered multiple signaling pathways in placenta and brain in the offspring of F2 and F3 generations. Our DNA methylation analysis revealed transgenerational changes in the DNA methylation at promoters of the genes that are important for the embryonic development.

The present data are in line with previous findings made by our laboratory using the same stress model. Investigation of the cumulative stress lineage animals in the F3 generation revealed reduced pup body weight and altered behaviour (Yao et al 2014). These findings were accompanied by the progressive decline in the gestational length with each generation leading to a higher risk of preterm birth (Yao et al 2014). We also demonstrated previously that stress exposure during pregnancy had a significant effect on maternal behaviour (Ward et al 2013). Animals from cumulative stress exposure groups in F1 and F2 generations showed a significant reduction in tail chasing behaviours when compared with controls (Ward et al 2013). Similar effects were observed in maternal behaviour in the non-cumulative stress exposure group in the F2 generation. Interestingly, no effect on tail chasing behaviour was found in stressed F0 mothers (Ward et al 2013). Our findings together with previous data suggest that stressful experiences during gestation can cause transgenerational changes in brain function and contribute to pathological conditions of the brain. However, it is important to note that it might be difficult to fully translate our findings with respect to the importance for human mental health. Critical causal mechanisms and phenotypes of stress observed in animal models have potential translational

limitations and may differ in their ecological validity. Nevertheless, given that the central components of the stress response is universal to all mammals and some of the epigenetic factors (e.g. miRNA structure) are conserved between rodents and humans, data from the rats may shed important light on the general mechanisms of transgenerational inheritance of stress responses. Overall, the results of the current study contribute to the existing examples of transgenerational epigenetic inheritance in different species and highlight that stressful events during gestation may have long-lasting effects on the progeny across multiple generations.

Ancestral stress during gestation causes transgenerational changes in the expression of genes important for normal embryonic development in placenta and embryonic cortex in the F3 offspring

Our results show that gestational stress in the parental (F0) generation altered multiple pathways in placenta and brain in F3 generation offspring. Transgenerational changes included disruption of the placental glucocorticoid barrier, blood-brain barrier, altered expression of imprinted genes and growth factors, neurotransmitters receptors and transporters, altered prostaglandin synthesis, synaptic plasticity and axon guidance in the embryonic cortex (see Chapter 2). All of these changes may contribute to the development of neurological and psychiatric pathologies later in life. Interestingly, our results showed that stress caused alteration in the gene pathways associated with the brain diseases, such as glioma, amyotrophic lateral sclerosis, prion disease, Huntington's and Alzheimer's diseases, once again highlighting a possible role of developmental programming of adult diseases.

Ancestral stress causes transgenerational changes in miRNA expression in placenta and embryonic cortex in the F3 offspring

Our results demonstrate that exposure of the great-grandmother to stress during gestation may alter important molecular pathways in the brain of great-grand offspring. These transgenerational effects of stress exposure might be regulated by epigenetic mechanisms. In particular, we demonstrated that miR-99b and miR-100 were up-regulated in both placenta and

embryonic cortex of the F3 progeny, whereas insulin-like growth factor 1 receptor (IGFR1), that is among confirmed miR-100 targets, was down-regulated on the protein level in the same tissues. Expression levels of miR-100 in embryonic cortex in the F3 offspring in the SNNN group also correlated with the *Igfr1* mRNA expression in placenta in the SNNN group. Our mRNA expression data showed multiple changes in the expression of insulin-like growth factors and other imprinted genes. This is in accordance with other findings from the literature, where stress and endocrine disruptors were shown to cause alterations in genomic imprinting (Drake et al 2011, Kang et al 2011, Susiarjo et al 2013). Thus, our results showed that measuring the levels of miRNA expression in placenta could provide a useful proxy to reflect the processes that happen in the brain of newborns, thus providing an opportunity for early diagnosis of brain diseases as well as opportunities for preventive interventions.

Ancestral stress causes transgenerational changes in DNA methylation of the genes important for embryonic development

Our results indicate that great-grand mother exposure to stress during gestation caused transgenerational changes in the DNA methylation at promoter regions of the transcription factor genes that are of particular importance for the normal development, such as *Atoh8* and *Deaf1*. However, we observed a lack of correlation between changes in the DNA methylation patterns and mRNA expression.

Overall, our results demonstrated that prenatal stress causes changes in gene and miRNA expression as well as DNA methylation in the brain and placenta. Consequences of prenatal stress applied to the parental generation can be transmitted to subsequent generations to influence lifetime health and disease trajectories.

Future studies

Several experiments may be performed to verify further role of epigenetic factors in transgenerational stress inheritance. Since in the current study we observed small effects of stress on the DNA methylation changes in either the placenta or embryonic brain it might be useful to

investigate the changes in other epigenetic components, like histone modifications in the same tissues. In addition, other tissues (e.g. germlines) could be used for further analysis, because they may be more prone to DNA methylation changes in response to stress. Alternative, increasing the sample size for the DNA methylation analysis could verify the reliability of the data from the current study.

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Supplemental materials

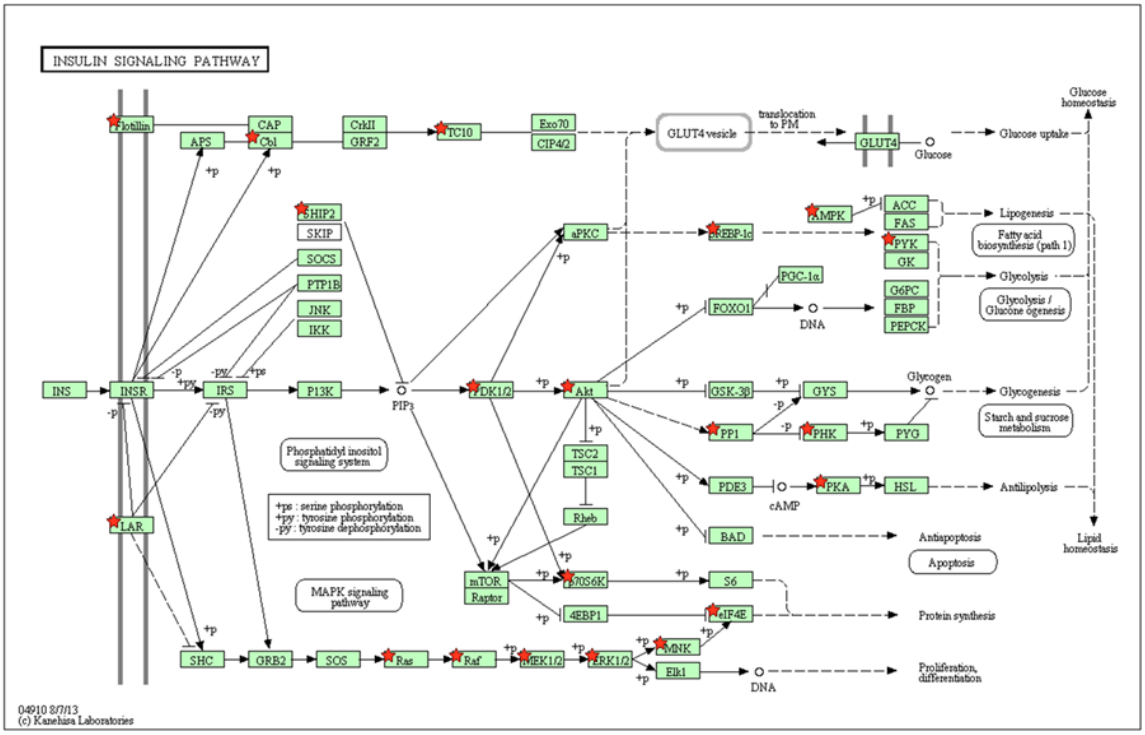

Figure S1. KEGG insulin signaling pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

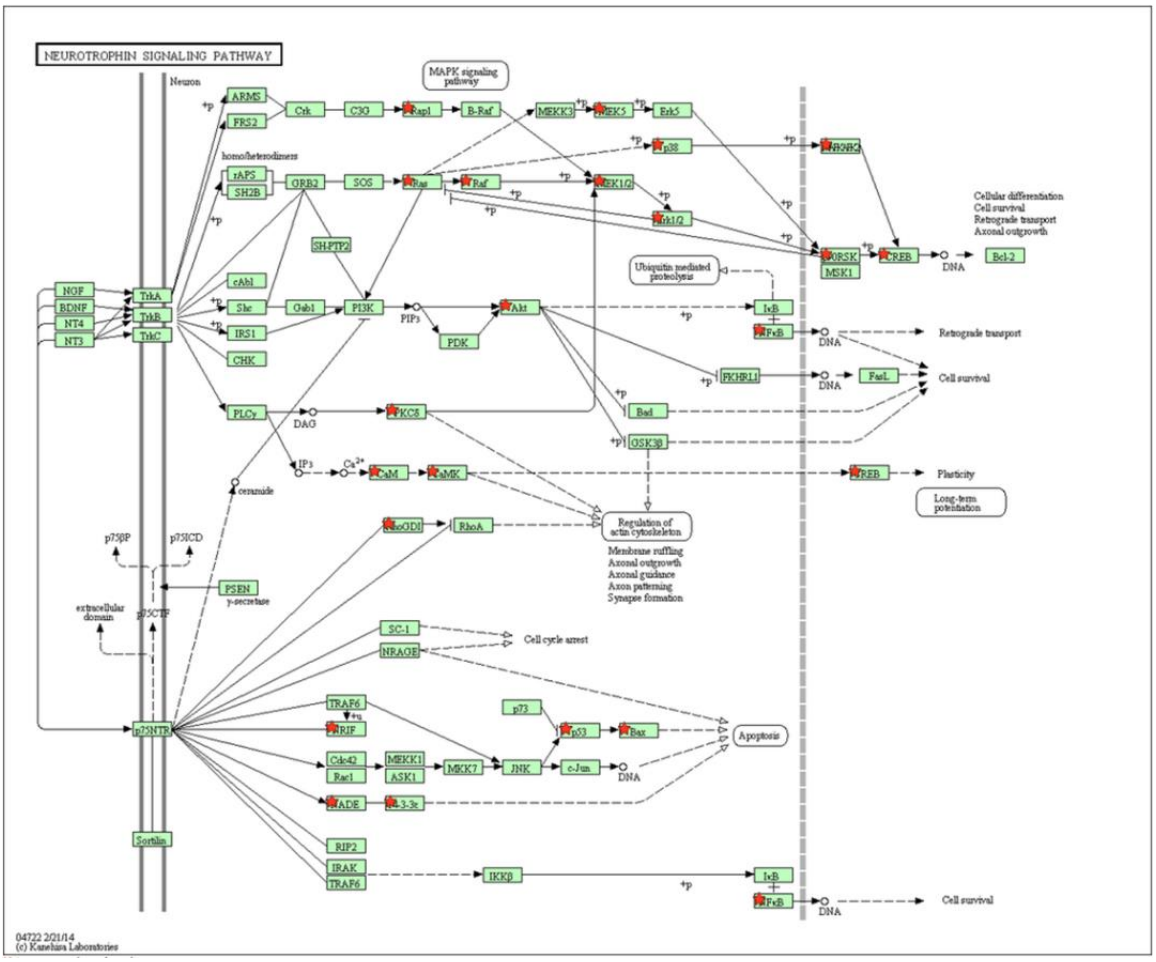

Figure S.2. KEGG neurotrophin signaling pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

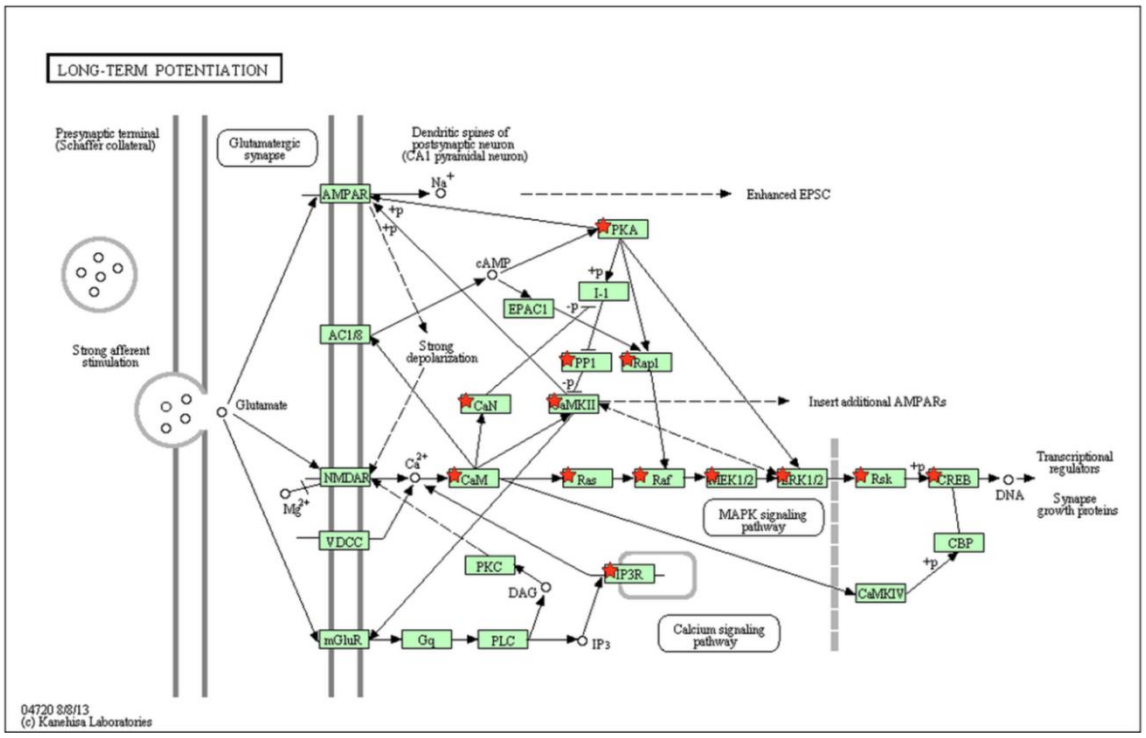

Figure S3. KEGG long-term potentiation pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

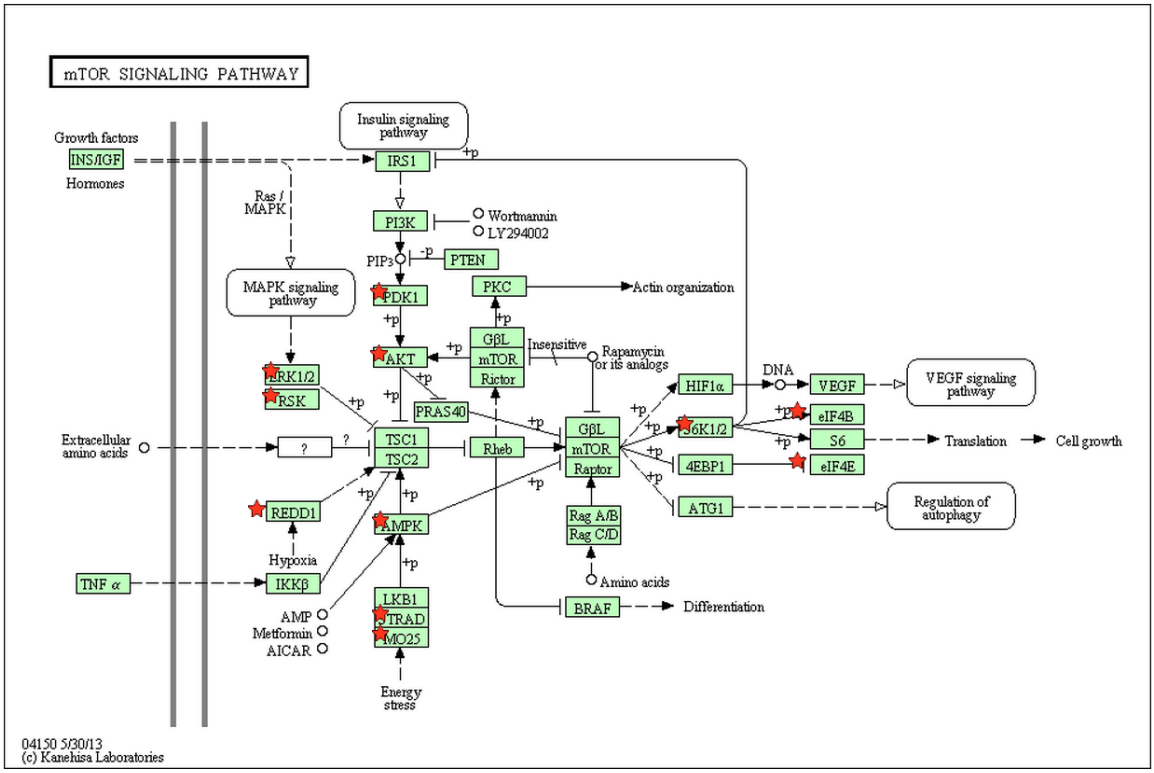

Figure S4. KEGG mTOR signaling pathway. Red stars indicate genes that were altered in response to stress (Kanehisa et al 2014).

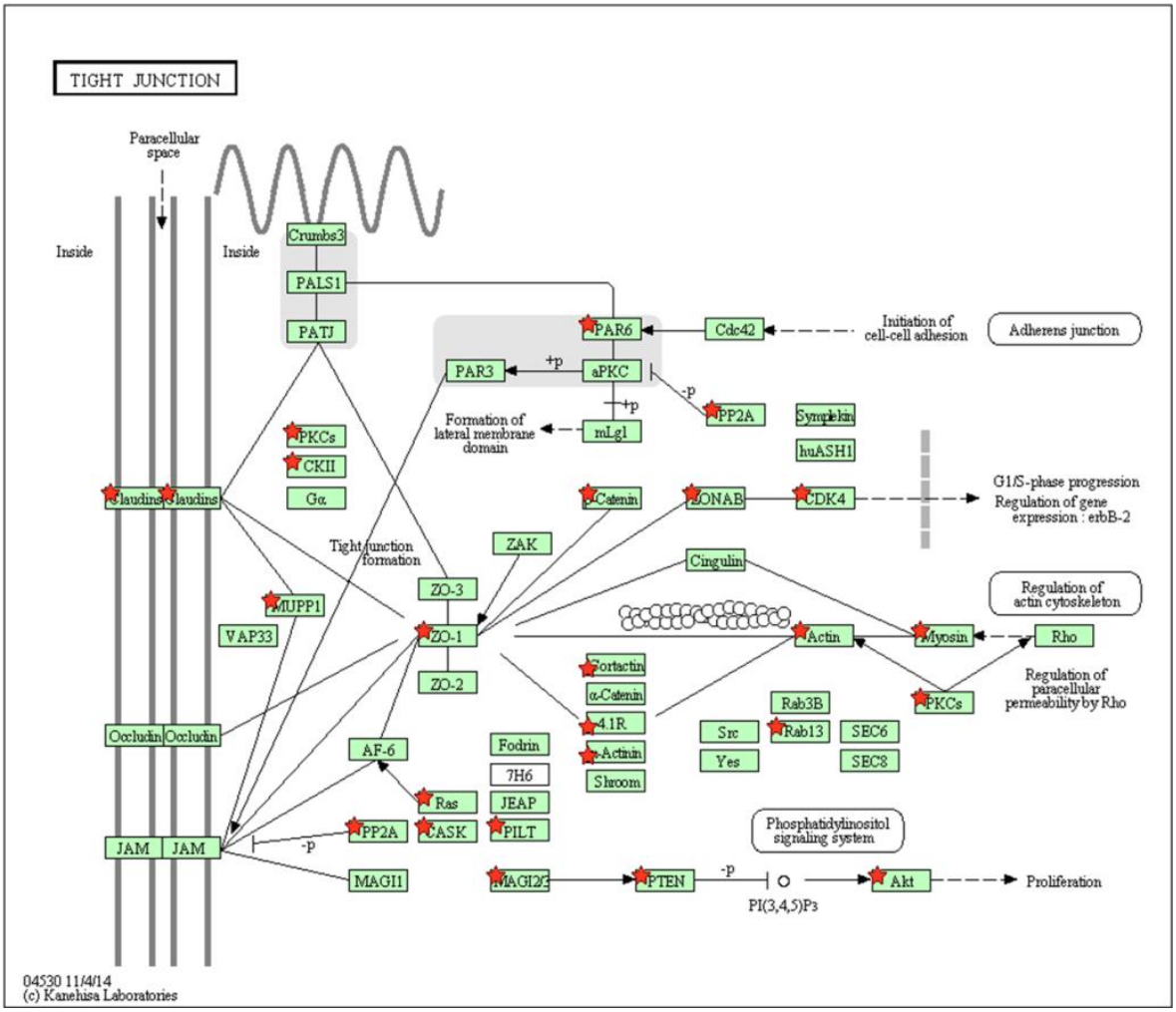

Figure S5. KEGG tight junction pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

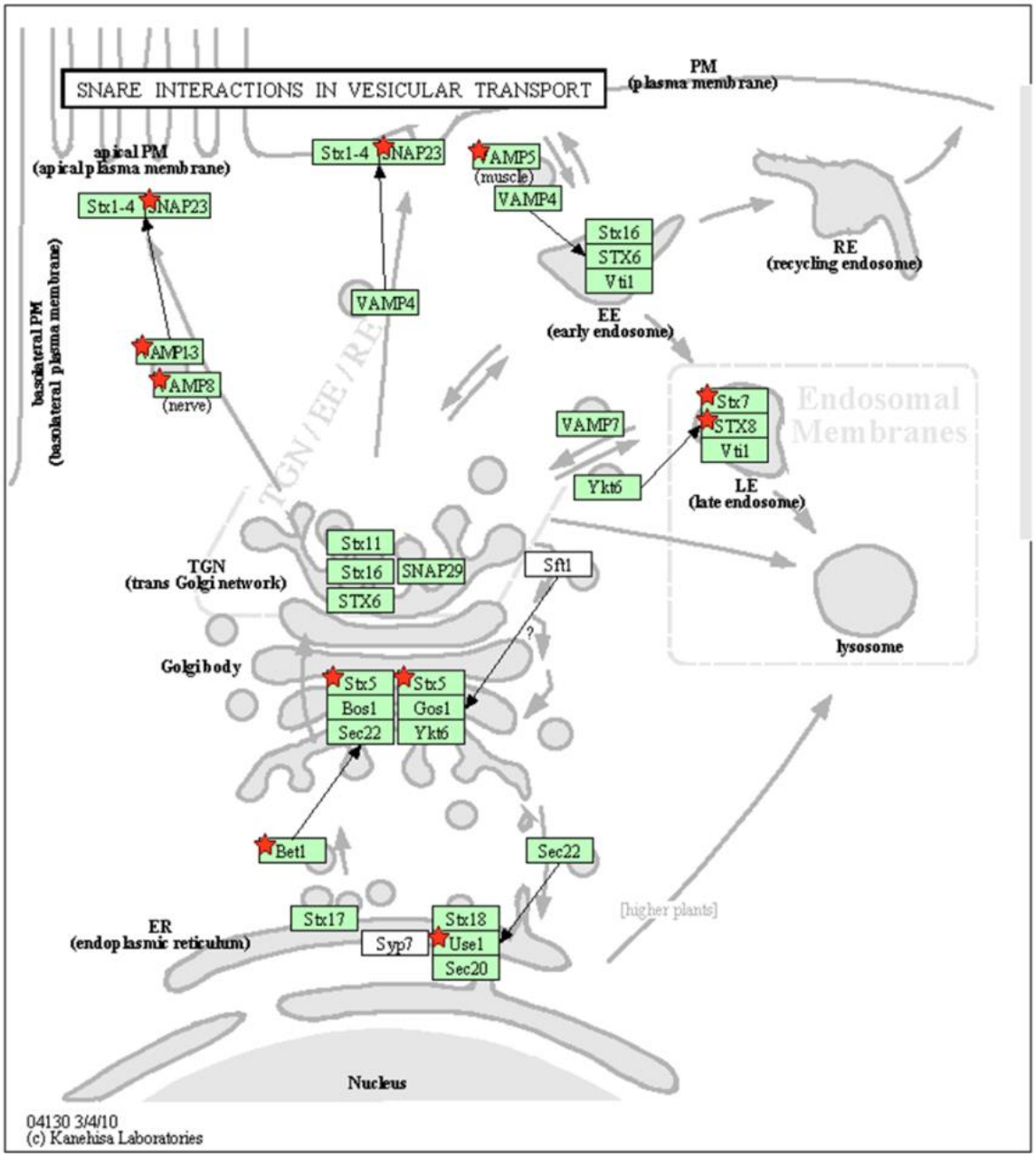

Figure S6. KEGG SNARE interactions in vesicular transport pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

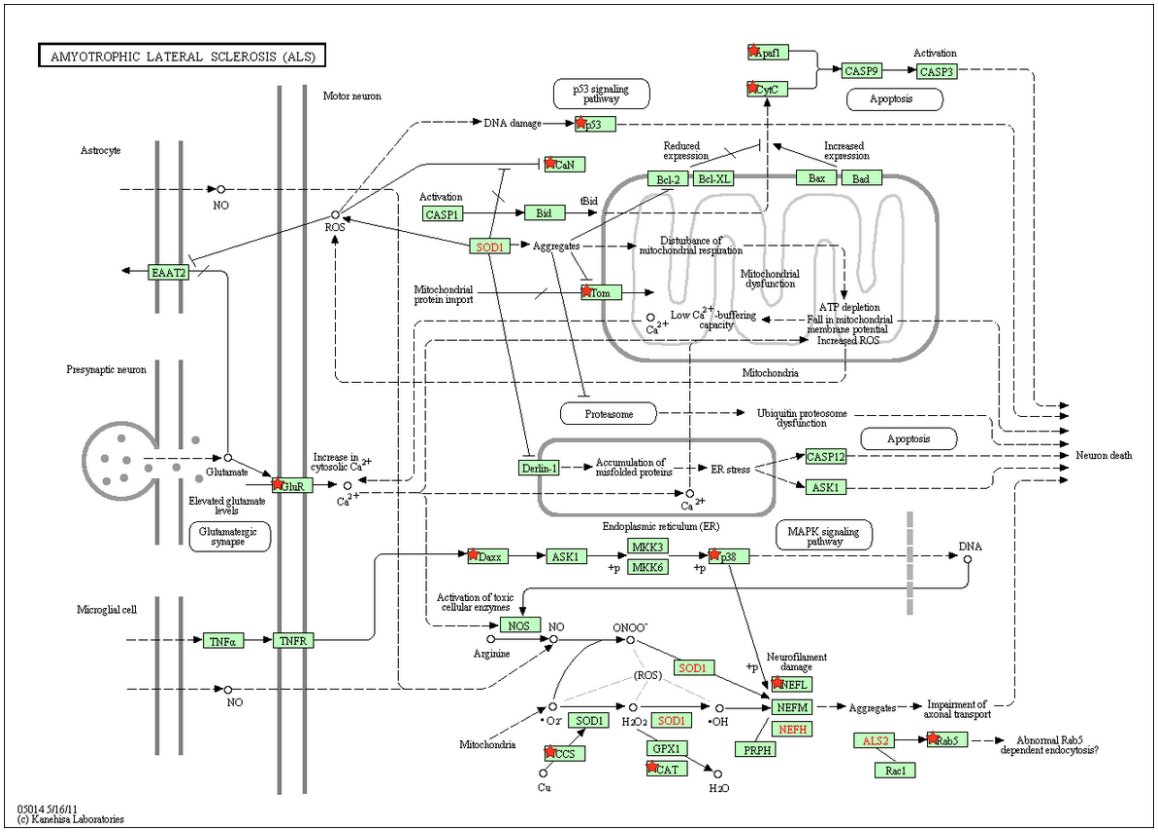

Figure S7. KEGG amyotrophic lateral sclerosis pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

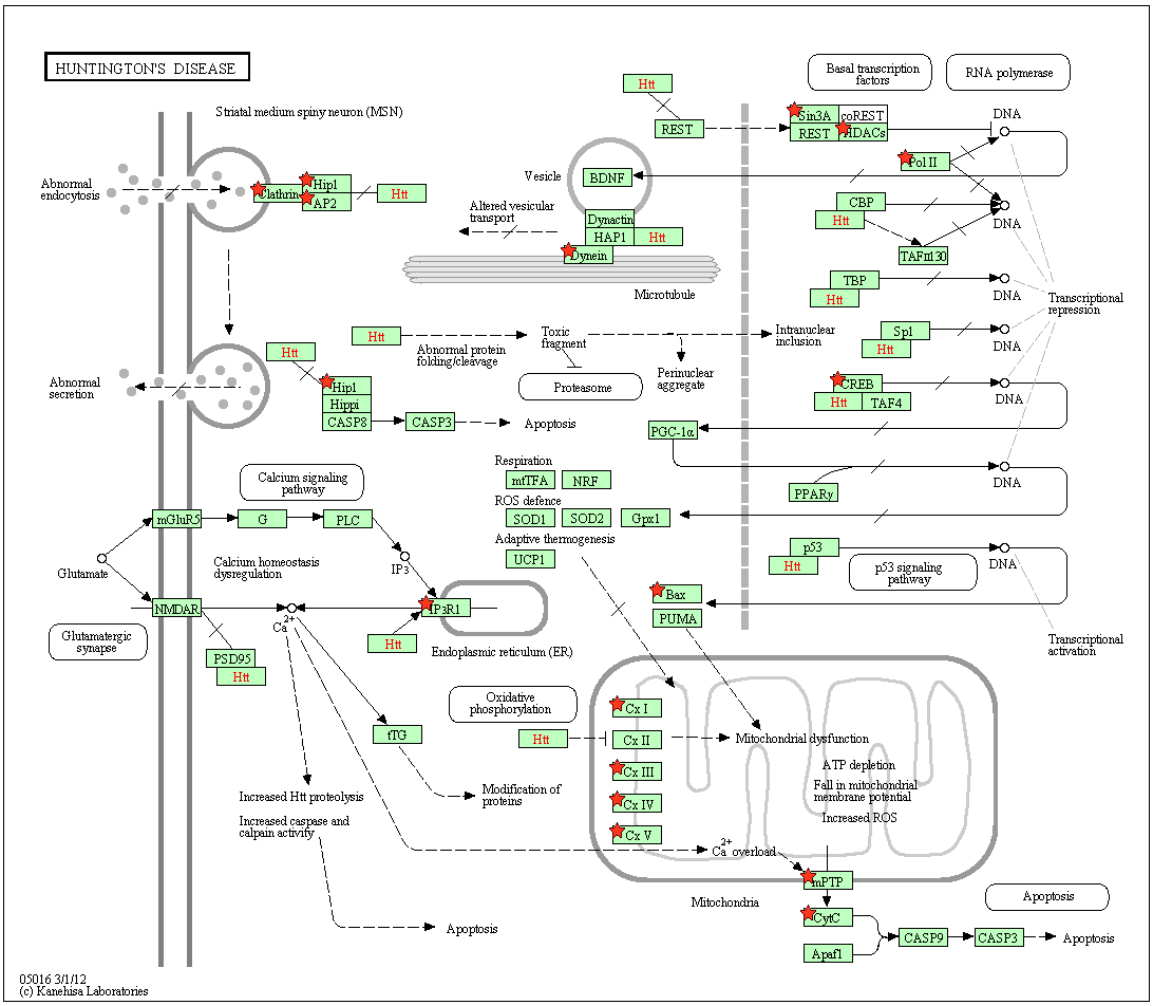

Figure S8. KEGG Huntington's disease pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

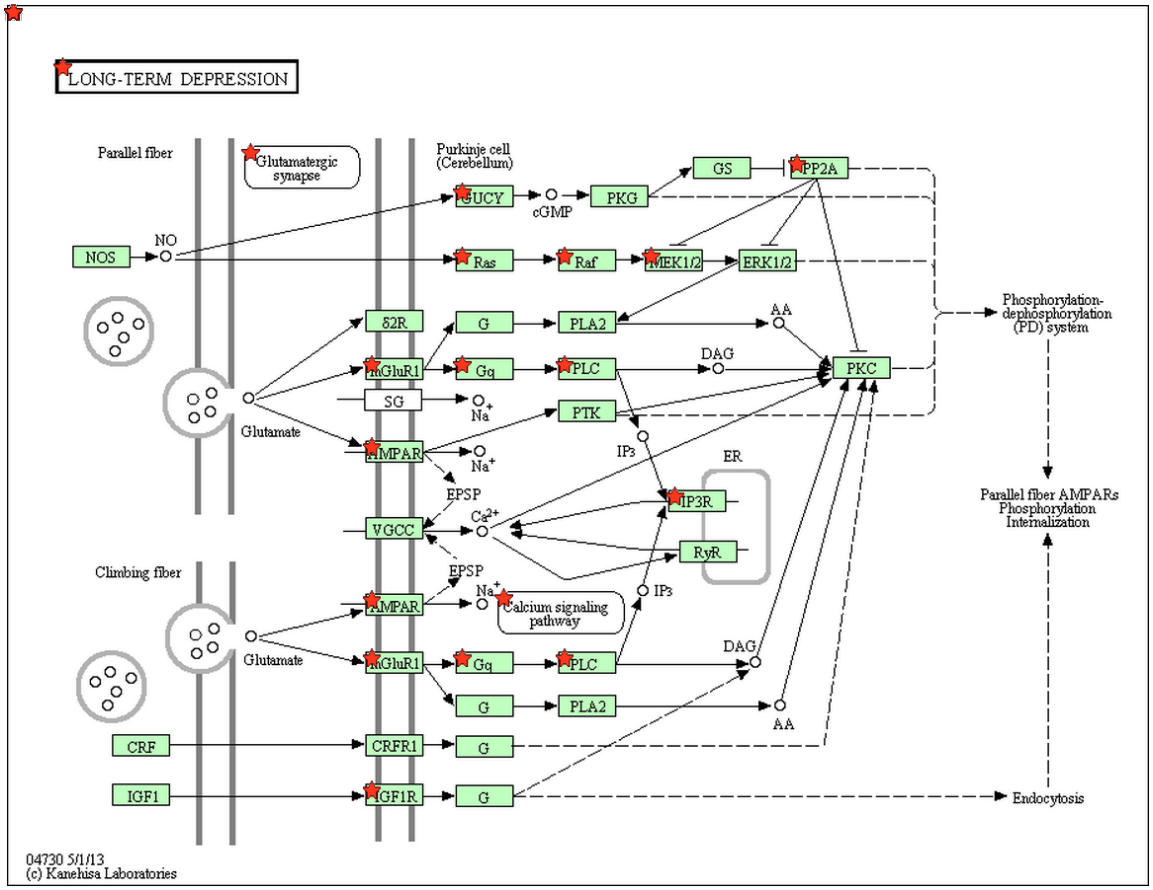

Figure S9. KEGG long-term depression pathway(Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

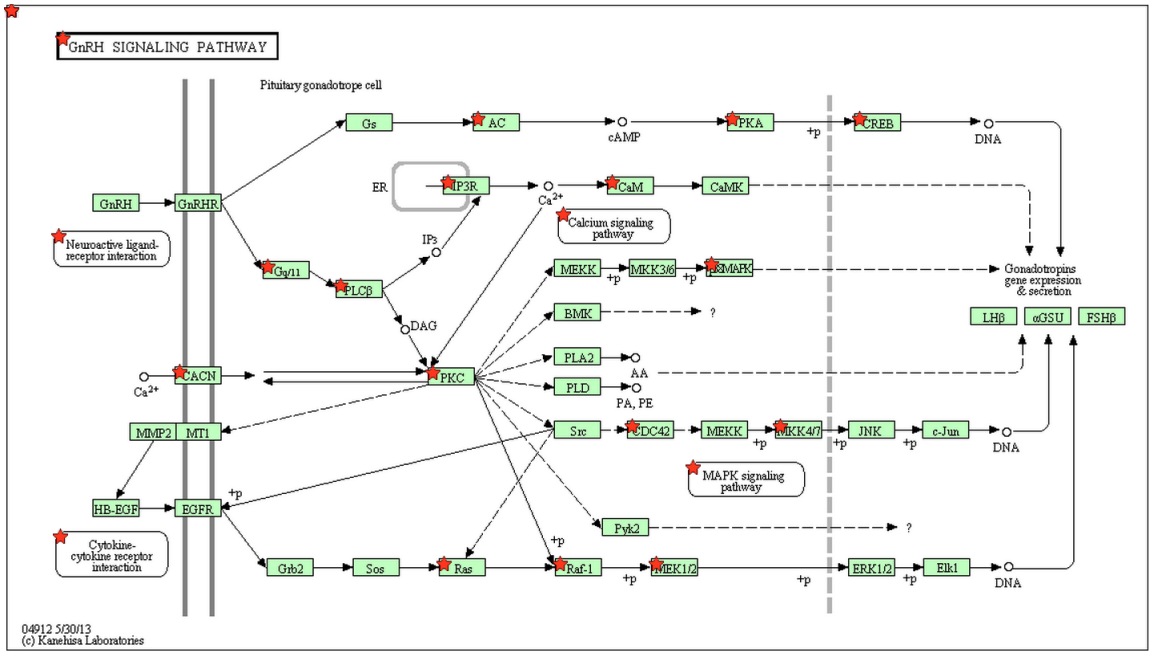

Figure S10. KEGG GnRH signaling pathway (Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

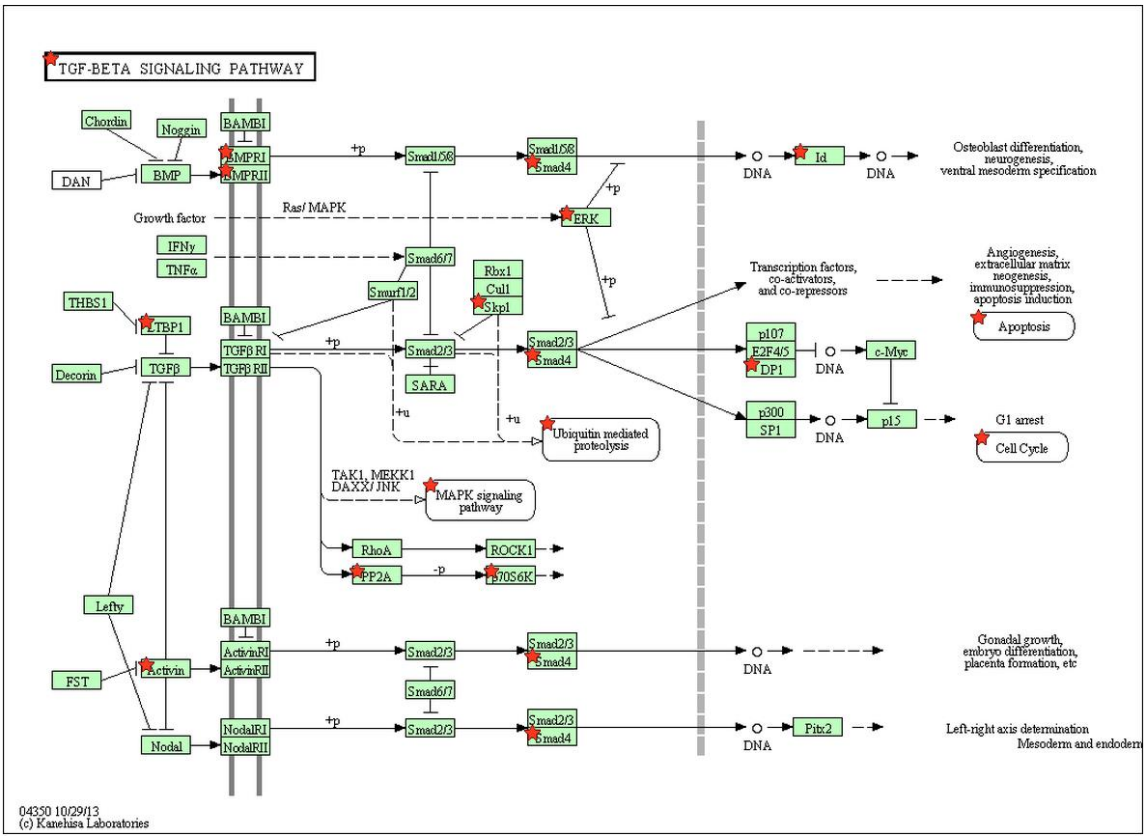

Figure S11. KEGG TGF-beta signaling pathway(Kanehisa et al 2014). Red stars indicate genes that were altered in response to stress.

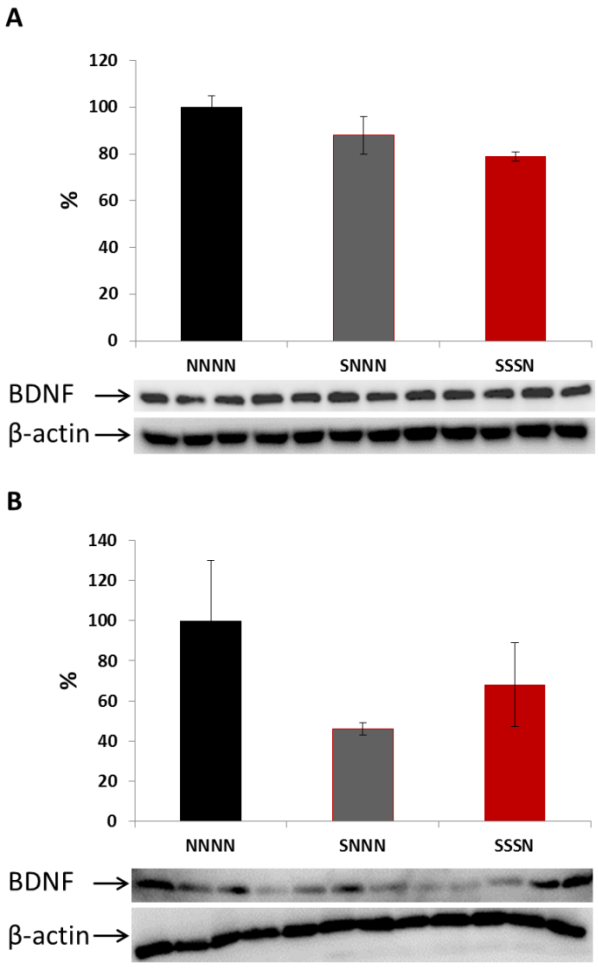

Figure S12. BDNF protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation.

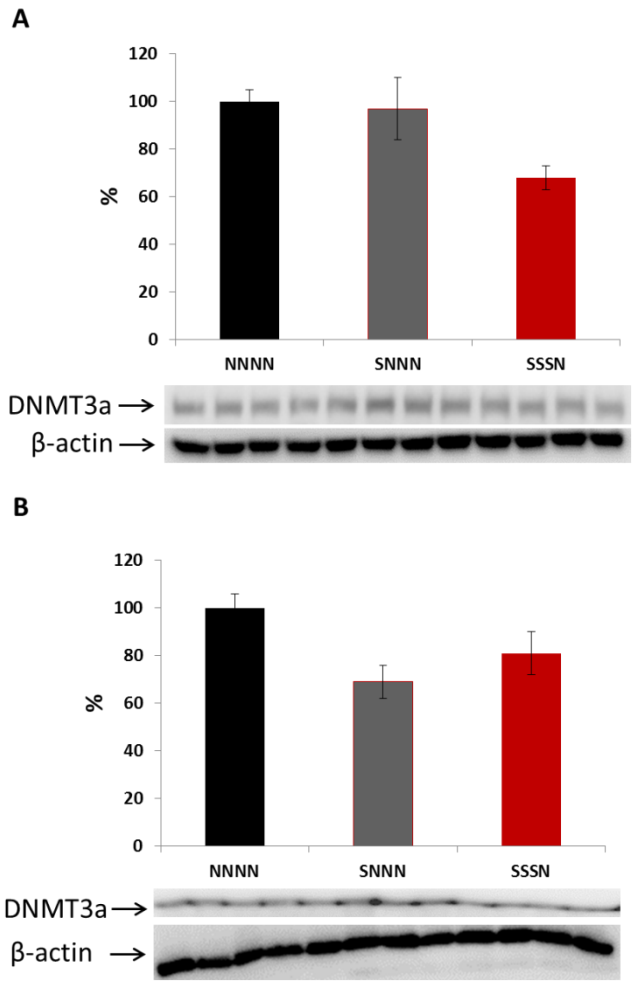

Figure S13. DNMT3a protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation.

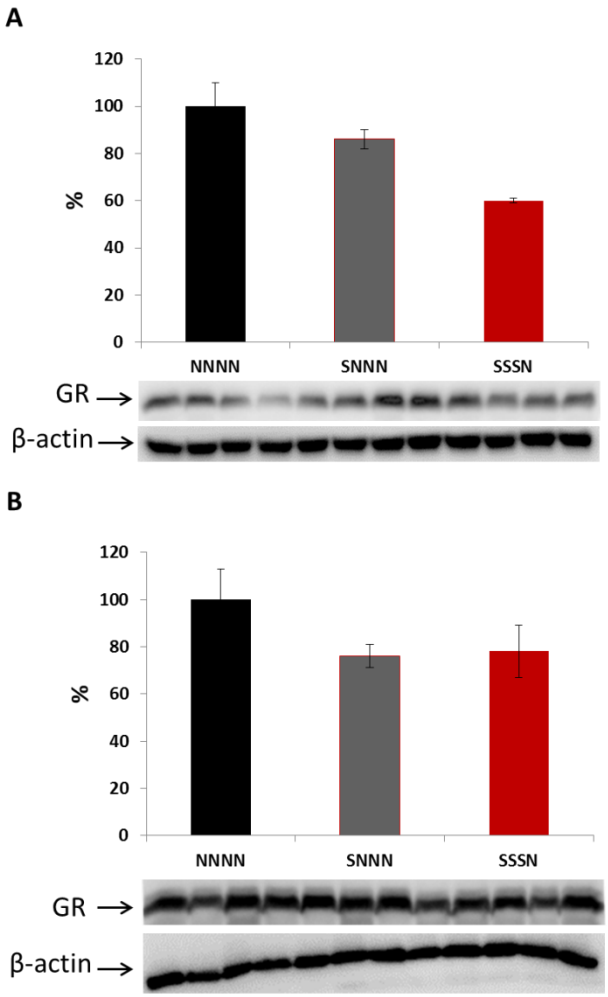

Figure S14. GR protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation.

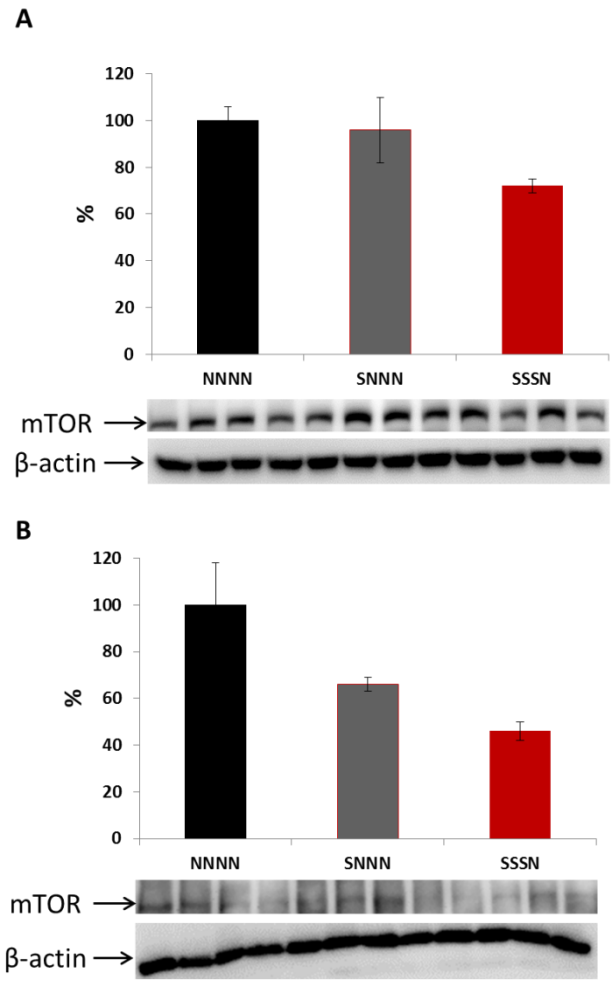

Figure S.15. mTOR protein expression (relative protein levels as % from control). A: E21 cortex, B: placenta. Data are shown as mean ± standard deviation (n=4).

Table S1. Annotation Clustering based on functional categories

Term	Count	%	P-Value	Fold Enrichment
phosphoprotein	634	25.1	0.000	1.3
acetylation	391	15.5	0.000	1.9
cytoplasm	351	13.9	0.000	1.4
nucleus	317	12.6	0.000	1.3
zinc	152	6	0.027	1.2
mitochondrion	91	3.6	0.013	1.3
zinc-finger	88	3.5	0.020	1.2
endoplasmic reticulum	87	3.4	0.004	1.3
golgi apparatus	71	2.8	0.001	1.4
protein transport	70	2.8	0.000	1.8
protein biosynthesis	66	2.6	0.000	2.9
ubl conjugation	64	2.5	0.003	1.4
rna-binding	63	2.5	0.000	1.9
ribonucleoprotein	60	2.4	0.000	2.5
transit peptide	60	2.4	0.005	1.4
ribosomal protein	55	2.2	0.000	2.7
cell cycle	55	2.2	0.000	1.9
ubl conjugation pathway	47	1.9	0.000	1.8
serine/threonine-protein kinase	44	1.7	0.047	1.3
Apoptosis	42	1.7	0.001	1.6
isopeptide bond	40	1.6	0.002	1.6
ribosome	39	1.5	0.000	3.3
methylation	39	1.5	0.000	1.8
mrna processing	29	1.1	0.002	1.8
mrna splicing	27	1.1	0.000	2.1
cell division	27	1.1	0.020	1.6
endosome	26	1	0.018	1.6
lipid metabolism	24	1	0.024	1.6
Chaperone	22	0.9	0.027	1.6
lysosome	22	0.9	0.034	1.6
tpr repeat	20	0.8	0.014	1.8
Isomerase	19	0.8	0.031	1.7
prenylation	19	0.8	0.031	1.7
Spliceosome	18	0.7	0.001	2.4
peroxisome	18	0.7	0.009	1.9
Initiation factor	17	0.7	0.000	3
er-golgi transport	17	0.7	0.001	2.3
proteasome	15	0.6	0.003	2.3

dna replication	12	0.5	0.017	2.1
serine/threonine-specific protein kinase	12	0.5	0.026	2
mRNA transport	12	0.5	0.031	2
translocation	12	0.5	0.037	1.9
blocked amino end	12	0.5	0.044	1.9
Redox-active center	10	0.4	0.026	2.2
sh3-binding	10	0.4	0.032	2.2
Hydrogen ion transport	10	0.4	0.039	2.1
stress response	10	0.4	0.039	2.1
homotetramer	7	0.3	0.015	3.1
autophagy	7	0.3	0.015	3.1
threonine protease	7	0.3	0.042	2.6
elongation factor	7	0.3	0.042	2.6
rrna-binding	6	0.2	0.031	3.1
dna-directed rna polymerase	6	0.2	0.031	3.1
growth arrest	5	0.2	0.015	4.5

Table S**2.** Gene classification based on gene ontology. Top 10 categories with the highest fold enrichment

Term	Count	%	P-Value	Fold Enrichment
regulation of amyloid precursor protein biosynthetic process	5	0.2	0.001	7.9
medium-chain fatty acid transport	3	0.1	0.044	7.9
smooth muscle cell migration	3	0.1	0.044	7.9
protein sumoylation	4	0.2	0.030	5.3
endoplasmic reticulum organization	5	0.2	0.012	4.9
estrogen receptor signaling pathway	4	0.2	0.048	4.5
ribosomal subunit assembly	4	0.2	0.048	4.5
regulation of translational initiation in response to stress	4	0.2	0.048	4.5
purine base biosynthetic process	4	0.2	0.048	4.5
regulation of translation in response to stress	4	0.2	0.048	4.5

Table S**3.** Annotation Clustering based on functional categories

Term	Count	%	P-Value	Fold Enrichment
acetylation	324	14.3	0.000	1.7
phosphoprotein	606	26.7	0.000	1.4
cytoplasm	320	14.1	0.000	1.4
protein biosynthesis	56	2.5	0.000	2.7
nucleus	303	13.4	0.000	1.4

ribonucleoprotein	51	2.2	0.000	2.3
ribosomal protein	45	2	0.000	2.4
ribosome	31	1.4	0.000	2.9
cell cycle	50	2.2	0.000	1.9
nucleotide-binding	184	8.1	0.000	1.3
protein transport	59	2.6	0.000	1.7
ubl conjugation pathway	43	1.9	0.000	1.8
ubl conjugation	65	2.9	0.000	1.6
atp-binding	140	6.2	0.000	1.3
methylation	37	1.6	0.000	1.9
Initiation factor	15	0.7	0.000	3
ligase	36	1.6	0.000	1.9
DNA damage	24	1.1	0.000	2.2
tumor suppressor	17	0.7	0.000	2.6
isopeptide bond	38	1.7	0.001	1.7
rna-binding	47	2.1	0.001	1.6
wd repeat	34	1.5	0.002	1.7
dna repair	20	0.9	0.002	2.1
dna replication	13	0.6	0.003	2.6
golgi apparatus	63	2.8	0.003	1.4
alternative splicing	150	6.6	0.003	1.2
mrna splicing	22	1	0.004	1.9
Spliceosome	15	0.7	0.005	2.2
Isomerase	20	0.9	0.005	1.9
myristate	16	0.7	0.008	2.1
mrna processing	25	1.1	0.008	1.7
serine/threonine-protein kinase	44	1.9	0.010	1.5
Transcription	109	4.8	0.010	1.2
mitochondrion	84	3.7	0.010	1.3
cell division	26	1.1	0.011	1.7
prenylation	19	0.8	0.012	1.8
phospholipid biosynthesis	9	0.4	0.016	2.6
magnesium	44	1.9	0.017	1.4
sterol biosynthesis	8	0.4	0.018	2.8
mitochondrion outer membrane	14	0.6	0.019	2
translocation	12	0.5	0.019	2.1
endosome	24	1.1	0.019	1.6
Cholesterol biosynthesis	7	0.3	0.019	3
gtp-binding	44	1.9	0.021	1.4
er-golgi transport	13	0.6	0.026	2
glycolysis	11	0.5	0.027	2.1

EF hand	11	0.5	0.027	2.1
coiled coil	119	5.2	0.029	1.2
transcription regulation	98	4.3	0.031	1.2
helicase	12	0.5	0.032	2
serine/threonine-specific protein kinase	11	0.5	0.032	2.1
Apoptosis	33	1.5	0.034	1.4
mRNA transport	11	0.5	0.039	2
cAMP binding	4	0.2	0.042	4.6
mitosis	16	0.7	0.045	1.7
Steroid biosynthesis	10	0.4	0.047	2
chromatin regulator	15	0.7	0.048	1.7
phosphotransferase	22	1	0.050	1.5

Table S4. MiRNA expression in cortex and placenta in three generations.

miRNA	comparison	cortex			Placenta		
		FC	P-value	P-adj	FC	P-value	P-adj
rno-miR-871*	SN vs. CT	N/A			N/A		
	SSN vs. CT	N/A			**1.1**	0.975	1.000
	SSN vs. SNN	N/A			**0.9**	0.469	1.000
	SNN vs. CT	0	1.000	1.000	**1.2**	0.468	0.987
	SSSN vs. CT	N/A			**2.4**	0.000	0.000
	SNNN vs. CT	N/A			**2.3**	0.000	0.004
	SSSN vs. SNNN	N/A			**1.0**	0.969	1.000
rno-miR-322*	SN vs. CT	**0.6**	0.647	1.000	**1.0**	0.963	1.000
	SSN vs. CT	**1.0**	0.710	1.000	**1.2**	0.531	1.000
	SSN vs. SNN	**1.2**	0.500	1.000	**0.8**	0.380	1.000
	SNN vs. CT	**0.9**	0.999	1.000	**1.4**	0.149	0.981
	SSSN vs. CT	**1.9**	0.164	0.961	**2.4**	0.000	0.000
	SNNN vs. CT	**1.4**	0.339	1.000	**2.3**	0.000	0.006
	SSSN vs. SNNN	**1.4**	0.620	1.000	**1.0**	0.793	1.000
rno-miR-331	SN vs. CT	**0.3**	0.240	1.000	**0.4**	0.026	1.000
	SSN vs. CT	**0.3**	0.014	1.000	**0.3**	0.001	0.074
	SSN vs. SNN	**1.3**	0.260	1.000	**1.0**	0.928	1.000
	SNN vs. CT	**0.2**	0.001	0.377	**0.3**	0.003	0.356
	SSSN vs. CT	**0.3**	0.000	0.000	**0.2**	0.000	0.001
	SNNN vs. CT	**0.4**	0.000	0.010	**0.2**	0.000	0.006
	SSSN vs. SNNN	**0.9**	0.355	1.000	**0.9**	0.679	1.000
rno-miR-675*	SN vs. CT	N/A			**0.8**	0.303	1.000
	SSN vs. CT	N/A			**0.5**	0.018	0.478

	SSN vs. SNN	N/A			**1.0**	0.980	1.000
	SNN vs. CT	0.000	1.000	1.000	**0.5**	0.042	0.981
	SSSN vs. CT	N/A			**0.4**	0.023	0.325
	SNNN vs. CT	N/A			**0.4**	0.000	0.011
	SSSN vs. SNNN	N/A			**1.1**	0.783	1.000
rno-miR-100	SN vs. CT	**0.7**	0.350	1.000	**0.9**	0.882	1.000
	SSN vs. CT	**1.4**	0.320	1.000	**1.0**	0.796	1.000
	SSN vs. SNN	**1.0**	0.930	1.000	**1.0**	0.956	1.000
	SNN vs. CT	**1.5**	0.217	1.000	**1.1**	0.729	1.000
	SSSN vs. CT	**1.8**	0.014	0.489	**1.9**	0.000	0.013
	SNNN vs. CT	**2.2**	0.001	0.097	**2.0**	0.000	0.011
	SSSN vs. SNNN	**0.8**	0.117	1.000	**0.9**	0.483	1.000
rno-miR-370	SN vs. CT	**2.2**	0.423	1.000	**1.3**	0.354	1.000
	SSN vs. CT	**0.6**	0.610	1.000	**1.7**	0.080	0.804
	SSN vs. SNN	**0.8**	0.994	1.000	**1.2**	0.798	1.000
	SNN vs. CT	**0.8**	0.506	1.000	**1.4**	0.140	0.981
	SSSN vs. CT	**1.2**	0.505	1.000	**0.6**	0.047	0.442
	SNNN vs. CT	**1.2**	0.596	1.000	**0.5**	0.002	0.080
	SSSN vs. SNNN	**1.0**	0.855	1.000	**1.3**	0.171	1.000
rno-miR-501*	SN vs. CT	**0.7**	0.496	1.000	**0.9**	0.979	1.000
	SSN vs. CT	**3.1**	0.111	1.000	**1.3**	0.795	1.000
	SSN vs. SNN	**1.3**	0.793	1.000	**0.5**	0.207	1.000
	SNN vs. CT	**2.5**	0.117	1.000	**2.6**	0.054	0.981
	SSSN vs. CT	**4.1**	0.028	0.575	**3.1**	0.007	0.138
	SNNN vs. CT	**4.8**	0.023	0.497	**3.7**	0.002	0.080
	SSSN vs. SNNN	**0.9**	0.758	1.000	**0.8**	0.609	1.000
rno-miR-153	SN vs. CT	**1.0**	0.858	1.000	**0.6**	0.615	1.000
	SSN vs. CT	**0.8**	0.554	1.000	**0.3**	0.328	0.963
	SSN vs. SNN	**1.0**	0.706	1.000	**0.4**	0.239	1.000
	SNN vs. CT	**0.8**	0.667	1.000	**0.9**	1.000	1.000
	SSSN vs. CT	**0.8**	0.344	1.000	**1.3**	0.484	0.898
	SNNN vs. CT	**0.8**	0.725	1.000	**3.7**	0.002	0.080
	SSSN vs. SNNN	**0.9**	0.656	1.000	**0.3**	0.038	1.000
rno-miR-181a	SN vs. CT	**0.9**	0.979	1.000	**1.0**	0.849	1.000
	SSN vs. CT	**1.0**	0.593	1.000	**0.9**	0.775	1.000
	SSN vs. SNN	**1.0**	0.694	1.000	**0.9**	0.333	1.000
	SNN vs. CT	**1.1**	0.462	1.000	**1.0**	0.902	1.000
	SSSN vs. CT	**1.3**	0.030	0.575	**1.6**	0.001	0.043
	SNNN vs. CT	**1.3**	0.079	0.903	**2.1**	0.003	0.099
	SSSN vs. SNNN	**1.0**	0.723	1.000	**0.7**	0.474	1.000

rno-miR-99b	SN vs. CT	**0.8**	0.367	1.000	**1.0**	0.884	1.000
	SSN vs. CT	**2.3**	0.024	1.000	**1.4**	0.043	0.769
	SSN vs. SNN	**1.1**	0.274	1.000	**0.9**	0.578	1.000
	SNN vs. CT	**2.1**	0.024	1.000	**1.6**	0.036	0.981
	SSSN vs. CT	**2.4**	0.004	0.310	**2.3**	0.000	0.000
	SNNN vs. CT	**3.2**	0.001	0.097	**2.5**	0.000	0.000
	SSSN vs. SNNN	**0.8**	0.123	1.000	**0.9**	0.554	1.000
rno-miR-409-5p	SN vs. CT	**1.9**	1.000	1.000	**1.3**	0.374	1.000
	SSN vs. CT	**22.0**	0.284	1.000	**6.3**	0.000	0.000
	SSN vs. SNN	**1.4**	1.000	1.000	**1.3**	0.435	1.000
	SNN vs. CT	**16.3**	0.186	1.000	**4.8**	0.000	0.000
	SSSN vs. CT	**16.4**	0.000	0.000	**12.0**	0.000	0.003
	SNNN vs. CT	**15.7**	0.000	0.000	**11.2**	0.000	0.006
	SSSN vs. SNNN	**1.1**	1.000	1.000	**1.1**	0.898	1.000
rno-miR-9	SN vs. CT	**0.8**	0.771	1.000	**1.1**	0.745	1.000
	SSN vs. CT	**1.0**	0.653	1.000	**0.3**	0.000	0.013
	SSN vs. SNN	**1.0**	0.885	1.000	**0.6**	0.038	1.000
	SNN vs. CT	**1.1**	0.550	1.000	**0.6**	0.048	0.981
	SSSN vs. CT	**1.3**	0.154	0.961	**1.1**	1.000	1.000
	SNNN vs. CT	**1.3**	0.251	1.000	**1.1**	0.899	1.000
	SSSN vs. SNNN	**1.0**	0.721	1.000	**0.9**	0.926	1.000
rno-miR-29a	SN vs. CT	**2.2**	0.191	1.000	**0.6**	0.290	1.000
	SSN vs. CT	**0.9**	0.884	1.000	**0.3**	0.000	0.016
	SSN vs. SNN	**1.3**	0.468	1.000	**0.6**	0.018	1.000
	SNN vs. CT	**0.7**	0.498	1.000	**0.5**	0.442	0.987
	SSSN vs. CT	**0.7**	0.257	0.966	**0.8**	0.173	0.636
	SNNN vs. CT	**0.7**	0.288	1.000	**1.2**	0.774	1.000
	SSSN vs. SNNN	**0.9**	0.971	1.000	**0.7**	0.329	1.000
rno-miR-493*	SN vs. CT	**2.9**	0.215	1.000	**1.1**	0.543	1.000
	SSN vs. CT	**2.5**	0.290	1.000	**3.1**	0.000	0.016
	SSN vs. SNN	**1.5**	0.747	1.000	**2.1**	0.024	1.000
	SNN vs. CT	**1.8**	0.405	1.000	**1.5**	0.110	0.981
	SSSN vs. CT	**2.4**	0.272	0.979	**1.3**	0.370	0.814
	SNNN vs. CT	**1.4**	0.557	1.000	**1.0**	0.965	1.000
	SSSN vs. SNNN	**1.7**	0.608	1.000	**1.3**	0.380	1.000
rno-miR-3596d	SN vs. CT	**0.8**	0.709	1.000	**1.2**	0.491	1.000
	SSN vs. CT	**0.6**	0.187	1.000	**0.5**	0.001	0.064
	SSN vs. SNN	**1.3**	0.334	1.000	**0.6**	0.023	1.000
	SNN vs. CT	**0.4**	0.032	1.000	**0.8**	0.650	0.987
	SSSN vs. CT	**0.8**	0.103	0.955	**0.8**	0.285	0.783

	SNNN vs. CT	**0.9**	0.523	1.000	**0.8**	0.148	0.864
	SSSN vs. SNNN	**0.9**	0.413	1.000	**1.1**	0.484	1.000
rno-miR-30e*	SN vs. NNN	**1.2**	0.620	1.000	**1.0**	0.972	1.000
	SSN vs. NNN	**1.9**	0.073	1.000	**1.4**	0.166	0.948
	SSN vs. SNN	**1.0**	0.512	1.000	**1.0**	0.885	1.000
	SNN vs. NNN	**2.0**	0.019	1.000	**1.5**	0.129	0.981
	SSSN vs. NNNN	**1.9**	0.005	0.311	**2.2**	0.000	0.001
	SNNN vs. NNNN	**1.8**	0.017	0.453	**1.9**	0.001	0.053
	SSSN vs. SNNN	**1.1**	0.449	1.000	**1.2**	0.493	1.000
rno-miR-154	SN vs. NNN	**0.5**	0.459	1.000	**0.9**	0.522	1.000
	SSN vs. NNN	**0.7**	0.823	1.000	**1.3**	0.504	1.000
	SSN vs. SNN	**1.0**	0.977	1.000	**0.9**	0.897	1.000
	SNN vs. NNN	**0.7**	0.735	1.000	**1.3**	0.377	0.987
	SSSN vs. NNNN	**0.4**	0.136	0.961	**0.4**	0.000	0.018
	SNNN vs. NNNN	**0.3**	0.051	0.695	**0.4**	0.009	0.187
	SSSN vs. SNNN	**0.5**	0.595	1.000	**0.8**	0.479	1.000

Table S5. MiRNA expression in placenta, E21 embryonic cortex (SSSN, SNNN), and P133 prefrontal cortex

miRNA	**group**	**comparison**	**cortex**			**placenta**		
			FC	**P-value**	**P-adj**	**FC**	**P-value**	**P-adj**
miR-219-2-3p	E21	SSSN vs. CT	N/A	N/A	N/A	N/A		
		SSSN vs. SNNN	0.82	1.000	N/A	N/A		
		SNNN vs. CT	n/a	n/a	n/a	N/A		
	P133	SSSS vs. CT	0.85	0.488	1.000	N/A		
		SSSS vs. SNNN	0.67	0.000	0.005			
		SNNN vs. CT	1.28	0.131	1.000			
miR-338-3p	E21	SSSN vs. CT	0.85	0.774	1.000	N/A		
		SSSN vs. SNNN	0.76	0.751	1.000	N/A		
		SNNN vs. CT	1.12	1.000	1.000	N/A		
	P133	SSSS vs. CT	0.81	0.205	0.986	N/A		
		SSSS vs. SNNN	0.65	0.000	0.005			
		SNNN vs. CT	1.27	0.078	1.000			
miR-219-	E21	SSSN vs. CT	N/A	N/A	N/A	N/A		

5p		SSSN vs. SNNN	0.81	1.000	1.000	N/A		
		SNNN vs. CT	N/A	N/A	N/A	N/A		
	P133	SSSS vs. CT	0.37	0.000	0.002	N/A		
		SSSS vs. SNNN	0.38	0.000	0.005			
		SNNN vs. CT	1.00	1.000	1.000			
miR-21-5p	E21	SSSN vs. CT	1.18	0.220	0.963	0.934	0.761	0.969
		SSSN vs. SNNN	1.03	0.816	1.000	0.863	0.497	1
		SNNN vs. CT	1.16	0.413	1.000	1.084	0.597	1
	P133	SSSS vs. CT	0.90	0.255	1.000	N/A		
		SSSS vs. SNNN	0.75	0.002	0.207			
		SNNN vs. CT	1.21	0.053	1.000			
miR-101a-3p	E21	SSSN vs. CT	0.72	0.711	1.000	1.373	0.829	0.999
		SSSN vs. SNNN	1.34	0.833	1.000	0.773	0.103	1
		SNNN vs. CT	0.54	0.502	1.000	4.692	0.179	0.901
	P133	SSSS vs. CT	0.75	0.000	0.010	N/A		
		SSSS vs. SNNN	0.87	0.038	1.000			
		SNNN vs. CT	0.87	0.092	1.000			
miR-33-5p	E21	SSSN vs. CT	0.44	0.057	0.783	0.590	0.473	0.891
		SSSN vs. SNNN	0.78	0.467	1.000	0.778	0.745	1
		SNNN vs. CT	0.58	0.227	1.000	0.758	0.741	1
	P133	SSSS vs. CT	0.47	0.000	0.010	N/A		
		SSSS vs. SNNN	0.70	0.064	1.000			
		SNNN vs. CT	0.68	0.031	0.957			
miR-92a-3p	E21	SSSN vs. CT	1.04	1.000	1.000	1.422	0.350	0.814
		SSSN vs. SNNN	1.27	0.205	1.000	1.431	0.223	1
		SNNN vs. CT	0.84	0.330	1.000	0.995	0.927	1
	P133	SSSS vs. CT	1.69	0.000	0.010	N/A		
		SSSS vs. SNNN	1.09	0.620	1.000			
		SNNN vs. CT	1.58	0.009	0.957			
miR-30a-5p	E21	SSSN vs. CT	0.94	0.514	1.000	0.980	0.814	0.999
		SSSN vs. SNNN	0.80	0.017	1.000	0.727	0.012	1
		SNNN vs. CT	1.19	0.200	1.000	1.345	0.090	0.736

	P133	SSSS vs. CT	0.76	0.000	0.025	N/A		
		SSSS vs. SNNN	0.80	0.005	0.395			
		SNNN vs. CT	0.97	0.655	1.000			
miR-128-3p	E21	SSSN vs. CT	1.55	0.430	1.000	4.454	0.411	0.836
		SSSN vs. SNNN	0.94	1.000	1.000	n/a	n/a	n/a
		SNNN vs. CT	1.67	0.342	1.000	2.198	0.709	1
	P133	SSSS vs. CT	1.22	0.002	0.087	N/A		
		SSSS vs. SNNN	1.17	0.592	1.000			
		SNNN vs. CT	1.03	0.015	0.957			
miR-136-5p	E21	SSSN vs. CT	0.90	0.669	1.000	0.724	0.034	0.395
		SSSN vs. SNNN	0.76	0.078	1.000	0.908	0.321	1
		SNNN vs. CT	1.20	0.305	1.000	0.793	0.321	1
	P133	SSSS vs. CT	0.70	0.002	0.087	N/A		
		SSSS vs. SNNN	0.89	0.359	1.000			
		SNNN vs. CT	0.80	0.071	1.000			
miR-30e-5p	E21	SSSN vs. CT	0.62	0.001	0.127	0.840	0.224	0.705
		SSSN vs. SNNN	0.80	0.051	1.000	0.852	0.257	1
		SNNN vs. CT	0.78	0.162	1.000	0.984	0.889	1
	P133	SSSS vs. CT	0.67	0.003	0.141	N/A		
		SSSS vs. SNNN	0.86	0.306	1.000			
		SNNN vs. CT	0.78	0.077	1.000			

Table S6. Function of miRNA differentially expressed in P133, according to PubMed

miRNA	miRNA function	reference (PMID)
miR-219-5p	Psychological stress may lead to the significant up-regulation of rno-miR-219-5p in rat myocardial tissues.	24669248
	Expression of hsa-miR-219-5p was negatively correlated with the expression of metallothionein 1F (MT1F) and tribbles homolog 3 (TRIB3) in cells exposed to AgNPs (silver nanoparticles).	24974767
	Role in obesity-induced steatohepatitis.	24249635
	Was down-regulated in glioblastoma, inhibited receptor tyrosine kinase pathway by targeting EGFR in glioblastoma.	23690991
	Might play important role in glioblastoma multiforme.	22829753

	Down-regulation of miR-29c-3p and miR-219-5p were found to be associated with advanced clinical stages of meningioma. High expression of miR-190a and low expression of miR-29c-3p and miR-219-5p correlated significantly with higher recurrence rates in meningioma patients.	22674195
	Role in breast cancer. Fifteen miRNAs, including miR-219-5p, were identified to be significantly differentially expressed between primary tumors and corresponding distant metastases.	22294488
	Was down-regulated in glioblastoma.	20711171
miR-101a-3p	May play a role as an endogenous modulator of glucocorticoid receptor expression in the adrenal gland.	22128032
	Was deregulated in mouse Sertoli cells exposed to nonylphenol (endocrine disrupting chemical).	21914226
miR-33-5p	Regulated cholesterol efflux and HDL biogenesis by decreasing the expression of ABC transporters, ABCA1 and ABCG1.	20466885, 20466882, 20566875
	Role in response to the toxic effects of silver nanoparticles (AgNPs).	24974767
	Was down-regulated in the rat model of temporal lobe epilepsy.	22998082
	Role in ischemic stroke etiology and pathology.	20841499
	Was down-regulated in the hippocampus of Down Syndrome model in mice (Ts65Dn).	20396359
miR-92a-3p	Role in tumour necrosis factor receptor-associated periodic syndrome (TRAPS).	24066048
miR-30a-5p	Down-regulation of miRNA-30a alleviated cerebral ischemic injury through enhancing Beclin 1-mediated autophagy.	24771295
	miR-30a down-regulation aggravated pressure overload-induced cardiomyocyte hypertrophy.	23660952
	Serum miR-210 and miR-30a expressions tended to revert to fetal levels in Chinese adult patients with chronic heart failure.	23660476
	Was overexpressed in glioma cell lines and glioma samples as compared to the normal brain tissues; its expression level was positively correlated with tumor grade of malignancy.	23606081
	Is a member of the miR-30a family involved in BDNF tuning expression. NPY modulated miR-30a-5p and BDNF in opposite direction in an *in vitro* model of Alzheimer disease.	23358924
	PRDM1 was directly targeted by miR-30a-5p and modulated the Wnt/β-catenin pathway in a Dkk1-dependent manner during glioma growth.	23348703
	A set of differentially expressed miRNAs, including miR-30a-5p, acted as post-transcriptional inhibitors of BDNF in prefrontal cortex.	18632683

miR-128-3p	Is a strong novel candidate oncogenic microRNA in T-cell acute lymphoblastic leukemia that targets the PHF6 tumor suppressor gene.	24895337
miR-136-5p	Circulating miRNA profiles provided a biomarker for severity of stroke outcomes associated with age and sex in a rat model; miR-136 was highly expressed exclusively in adult females compared with middle-aged females, adult males and middle-aged males.	24428837
	Five tumor suppressor miRNAs (hsa-miR-136, -145, -342, -129, -376a) showed significant under-expression in clinical glioblastoma multiforme (GBM) tumor samples from the TCGA GBM cohort.	23577178
	Role in in human non-small cell lung cancer.	23959478
	Was up-regulated in mice after ovariectomy.	23929739
	Role in glioblastoma.	24705102
	Was differentially expressed between metastatic and non-metastattic giant cell tumor of bone.	23172052
	Was down-regulated in human glioma, and that promoted apoptosis of glioma cells induced by chemotherapy.	22967897
	Role in germ cell development. Was up-regulated between gonocytes and spermatogonia.	22536405
	Role in differentiation of stem cells.	19523330
miR-30e-5p	Expression was altered in the hypothalamus in response to caloric restriction and/or a high-fat diet.	24517225
	Role in radial glia cell proliferation; MiR-30e and miR-181d were identified as posttranscriptional negative regulators of HtrA1 by binding to its 3' untranslated region. *In vivo* overexpression of miR-30e and miR-181d in Dicer(-/-) forebrain rescued RG proliferation defects.	22854828
	There was an age-dependent decreased expression of microRNAs mmu-miR-181a-1*, mmu-miR-30e and mmu-miR-34a in the brain of calorie-restricted mice. Down-regulation of miR-34a, -30e, and -181a permitted their shared target gene expression (Bcl-2) to remain at a high level without post-transcriptional repression, accompanied by concomitant low levels of Bax expression and Caspase cleaving.	21415464
	Was among down-regulated miRNAs in fetal brains with prenatal ethanol exposure.	21415464
	Role in pathogenesis of schizophrenia, its expression in plasma of schizophrenia patient was higher than in normal controls.	24694668
	Role in gestational diabetes.	24279768
miR-219-2-3p	MicroRNA-219-2-3p functions as a tumor suppressor in gastric cancer and is regulated by DNA methylation.	23637748
	Role in acute inflammation.	22957142

	Was expressed in both rodent and human oligodendrocyte; may regulate myelination program in humans.	22470405
	Was aberrantly expressed in serum of schizophrenia patients	22094284
	Was decreased in diabetic retinopathy in rats.	22156553
miR-338-3p	Role in sporadic amyotrophic lateral sclerosis.	22903028
	Role in maturation of rat hippocampal neurons.	22363537
	Role in prion induced neurodegeneration; down-regulated in in the brains of mice infected with mouse-adapted scrapie.	18987751
	Was altered in early-stage estrogen receptor-positive breast cancer resection in post-menopausal women.	25004125
	Role in atrial myocardium during coronary bypass surgery.	24371264
	Suppressed neuroblastoma proliferation, invasion and migration through targeting PREX2a.	24140344
	Suppressed cell growth of human colorectal carcinoma.	23599646, 24277750
	Was altered in in the serum of patients with high-risk oral lesions.	23342275
	β cell mass expansion during pregnancy and obesity was associated with changes in the expression of several islet microRNAs, including miR-338-3p, in rodents. Blockade of miR-338-3p in β cells using specific anti-miR molecules mimicked gene expression changes occurring during β cell mass expansion and resulted in increased proliferation and improved survival both in vitro and in vivo.	22996663
miR-21-5p	Role in traumatic brain injury; was associated with the recovery process.	24920273, 19501075
	Role in neuronal maintenance during hibernation in bats.	24768722
	Role in in cerebral ischemia reperfusion injury.	24696166
	Contributed to chemoresistance of glioblastoma multiforme.	24690174
	Role in glioma; was increased in brain tumors of different grades.	24412320
	Was up-regulated in in glioblastoma.	24412053
	Was up-regulated in glioma tissues compared to the corresponding non-neoplastic brain tissues; its expression was associated with overall survival in patients with glioma; miR-21 was located in both tumor cells and tumor blood vessels and that its level in the tumor cell compartment had unfavorable prognostic value in gliomas; was demonstrated to be one of the major players in radio-resistance; miRNA-21 was developmentally regulated in mouse brain and was co-	24326156, 23212403, 23174013, 23104517, 23077620, 22964638, 22931209, 22766763

	expressed with SOX2 in glioma.	
	Role in glioblastoma; over-expression of miR-21 resulted in radiation resistance, while knockdown of miR-21 led to higher sensitivity of glioblastma cells to radiation.	23904372, 24012640, 23482671, 23314342, 23215807, 23201752
	Potential biomarker for cerebrovascular disease; there was a 6.2-fold increase for stroke risk when miR-21 levels increase.	23860376
	Serum miR-21 was a diagnostic and prognostic marker of primary central nervous system lymphoma.	23832112
	Role in spontaneous recovery in embolic stroke model; was significantly up-regulated during stroke recovery.	23823624
	Was up-regulated in the rat hippocampus following cerebral ischemia.	23473787
	Role in mesial temporal lobe epilepsy.	23315173
	Was up-regulated following spinal cord injury; regulated astrocytic response following spinal cord injury.	23238710
	Exosomes released by prion-infected neuronal cells had increased let-7b, let-7i, miR-128a, miR-21, miR-222, miR-29b, miR-342-3p and miR-424 levels.	22965126
	Was overexpressed in patients with poor neurological outcome after cardiac arrest.	22890253
	Role in ischemic injury.	21373187
	Was significantly up-regulated in the brain in both HIV associated dementia in humans and its monkey model SIV encephalitis.	21170291

Table S7. Genes differentially methylated at promoter regions in placenta

Generation	**Comparison/ Gene symbol**		**Methylation difference, %**	**Direction of change**
F1	**SN vs. CT**	Sstr5	-37	down
		Marcks	62	up
		Fgd1	71	
		Olfml2b	65	
		Smc6	48	
		Smc6	60	
		Nf2	44	
		Adcy1	35	
		Lad1	69	
		Espl1	51	
		Rnaset2	41	

		n/a	70	
		Higd2a	42	
		Zyx	52	
		Mtss1l	62	
		Ybey	54	
		Sox11	62	
		n/a	39	
		Pde2a	47	
		Mad2l2	50	
		Ly6h	35	
		Cacna1a	58	
F2	**SNN vs. CT**	ENSRNOT00000045515	-7	down
		ENSRNOT00000026765	12	up
		CD79A	43	
	SSN vs. CT	Novel pseudogene	-48	down
		Crkl	56	up
		Pds5a	44	
		Slc9a3r1	64	
		n/a	58	
		Tmem158	23	
		Psmd6	64	
		Nuak1	72	
		Kpnb1	36	
		Slc6a6	53	
		Sqle	70	
		n/a	38	
		Kcnk3	60	
		Cib2	66	
		Ccno	60	
		Api5	50	
		Ttc14	44	
		Snapc3	55	
		Xrn2	43	
		Frmpd1	26	
		n/a	66	
		n/a	42	
		Zfp131	56	
		Utp15	42	
		Wwtr1	53	
		Cib2	66	
		Ccno	60	

		Api5	50	
		Ttc14	44	
		Snapc3	55	
		Xrn2	43	
		Frmpd1	26	
		n/a	66	
		n/a	42	
		Zfp131	56	
		Utp15	42	
		Wwtr1	53	
		Ppargc1b	57	
		LOC100912286	61	
		Fgf4	55	
		Lrig3 protein	70	
		Ccdc58	35	
		Kcnh3	65	
		Trmt2a	77	
		Epha6	41	
		Syt7	65	
		Dcaf11	56	
		Pm20d2	55	
		Cplx1	70	
		n/a	54	
		n/a	48	
		Tmf1-201	44	
	SSN vs. SNN	Slc9a2	-49	down
		Bud31	66	up
		Lrrc59	57	
		Hk2	21	
		Spc25	60	
		Klrg2	74	
		Mtmr14	71	
		Car2	53	
		Zfp703	36	
		LOC100360552	57	
		Pcsk7	47	
		Lrif1	53	
		Mpv17l2	59	
		Homer3	60	
		Pias3	67	
		Tfap2b	77	

		Rbbp5	40	
		Prr12	50	
		Pax2	39	
		Pacs2	56	
		Tmeff1	72	
F3	**SNNN vs. CT**	Amh	-63	down
		Nrg1	27	up
		Atoh8	73	
		Zfp704	47	
		Npr1	66	
		Fzd1	29	
		Trim33	72	
		Cyp2u1	48	
		Btbd1	66	
		Cdc42	44	
		Lrig3	48	
		Slc25a10	54	
		N/A	39	
		N/A	52	
		N/A	53	
		N/A	65	
	SSSN vs. CT	Derl1	-54	down
		Actl6b	27	up
		Bfsp1	35	
		Npw	61	
		Gnaq	46	
		Nup133	53	
		N/A	40	
		N/A	49	
	SNNN vs. SSSN	Syn2	44	up
		ST8SIA2	65	
		MKI67	41	
		Carm1	46	
		rno-mir-9b-1	32	
		Phf12	34	
F2 vs F3	**NNN vs. NNNN**	Pofut2	-48	down
		Nrde2	-23	
		Rbm7	-33	
		Kctd5	-55	
		B3gat1	-76	
		Insm2	-12	

		Zfp800	-41	
		Zbtb8a	-53	
		Ccno	-60	
		Ttc14	-78	
		Rcn1	-70	
		Lmo1	-33	
		Hspa14	-40	
		March4	-41	
		Prmt6	-75	
		Sfrp1	-60	
		Asb13	-64	
		Ola1	-42	
		Eif1ad	-56	
		Rnaseh2c	-33	
		Cacna1h	-59	
		RGD1304704	-49	
		Grik1	-54	
		Cacna1a	-57	
		Cacna1a	-60	
		n/a	-21	
		n/a	-46	
		Hck	35	up
		Hes3	40	

Table S8. Genes differentially methylated at promoter regions in embryonic cortex

Generation	**Comparison/ Gene symbol**		**Methylation difference, %**	**Direction of change**
F1	**SN vs. CT**	ENSRNOT00000050876	31	up
F2	**SNN vs. CT**	Nr4a2	-53	down
		Ndufb11	-33	
		Zfp644	53	up
		Camta2	59	
		Tbx18	41	
		Plcl2	40	
		Epc1	69	
		Ctgf	41	
		Snta1	41	
		N/A	56	
		N/A	82	
		N/A	52	

	SSN vs. CT	Rbm18	37	up
		Fdx1	21	
		Hspa9	66	
		Tsen2	38	
		N/A	43	
	SSN vs. SNN	LOC316820	-55	down
		Parp1	36	up
		Dnaja1	73	
		Prmt3	100	
		Isyna1	61	
		Sgms1	64	
		LOC690899	51	
F3	**SNNN vs. CT**	Lin9	70	up
		Pp1	65	
		Deaf1	31	
		Zcchc14	71	
		Phtf2	47	
	SSSN vs. CT	Arhgef17	61	up
	SNNN vs. SSSN	Ajuba LIM protein	76	up
		Pde5a	56	
		Ubn2	63	
F2 vs. F3	**NNN vs. NNNN**	Kbtbd6	-67	down
		Ptma	-60	
		Polg	-37	