THE MOLECULAR MECHANISMS UNDERLYING EPIGENETICS OF THE STRESS RESPONSE IN THE CEREBELLUM IN A RAT MODEL

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Thesis Abstract

Previous findings showed that mild chronic restraint stress causes motor impairments in rats. These behavioural impairments might be related to molecular changes in brain areas that regulate motor functions, such as the cerebellum. Little is known about the role of the cerebellum in stress-induced behavioural alteration. We hypothesized that alteration in animal behaviour after chronic restraint stress is due to brain-specific changes in miRNA and proteins encoding gene expression. Our results revealed that expression of three miRNAs and 39 mRNAs was changed significantly after two weeks of stress. Furthermore, we verified one putative target for one of the changed miRNAs and expression of four randomly selected genes. Changes in gene expression disappeared after two weeks of recovery from stress. These findings provide a novel insight into stress-related mechanisms of gene expression underlying altered behavioural performance. The observations bear implications for the prevention and treatment of stress-related disorders and disease.

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List of Abbreviations

cAMP - cyclic adenosine monophosphate

HPA - hipotalamic-pituitary-adrenal

ACTH - adrenocorticotrophic hormone

CRH - corticotropin-releasing hormone

AVP - arginine-vasopressin

PVN - paraventricular nucleus

CORT - cortisol in primates and corticosterone in most rodents

SGP - slow growth period

AD - Alzheimer's disease

PD - Parkinson's disease

HD - Huntington's disease

MS - multiple sclerosis

MBD - methyl-CpG binding protein

DNMT - DNA methyltransferase

dNTP - deoxyribonucleotide triphosphate

CNS – central nervous system

ADP - adenosine diphosphate

SUMO - Small Ubiquitin-like Modifier

HAT - histone acetyltransferases

HDAC - histone deacetylases

HMT - histone methyltransferases

2WSTRESS – two week stress

2WCONTROL – two week control

4WSTRESS – four week stress

4WCONTROL – four week control

EIA - Enzyme Immunoassay

NSB - Non-Specific Binding

TA - Total Activity

Blk - Blank

UTR - untranslated region

PCR – polymerase chain reaction

TAE – Tris/Acetate/EDTA buffer

EDTA - ethylenediaminetetraacetic acid

LB - lysogeny broth

ANOVA - Analysis of Variance

LOWESS - Locally-weighted Regression

SD – Standard Deviation

Sq-RT-PCR – semi-quantitative reverse-transcription PCR

CDS - coding sequence

BBB - blood brain barrier

CSF - cerebrospinal fluid

PRLR - prolactin receptor

PRL - prolactin

GABA - γ-Aminobutyric acid

EGF - epidermal growth factor

INTRODUCTION

Although the term physiological stress is ubiquitously used today, it was not until 1914 when Walter Bradford Cannon first described the psychophysiology of the stress response (Quick and Spielberger, 1994). Cannon pioneered the study of stress by investigating the alarm and emergency components of the stress response and the role of the sympathetic nervous system in adaptation (Neylan, 1998). Cannon defined stress as "the body's ability to prepare itself instantaneously to respond to physical threat" (Cannon, 1914). A major contribution to the empiric paradigm of stress was made by Hans Selye, who described his concept of the General Adaptation Syndrome in 1936 (Selye, 1936). In this work Selye provided a description of physiological and morphological changes in the adrenal gland, the thymus, spleen and other lymphoid tissue, and gastric ulcerations, which were due to the response of an organism to the negative (distress) or positive (eustress) aspects of stress (Selye, 1936). It was actually Selye who introduced the term stress from engineering to describe this complex phenomenon. There are many definitions of stress, but most of them originate from the so-called classical definition provided by Selye (Selye, 1936). According to Selye, stress is defined as "the nonspecific response of the body to any demand made on it" (Selye, 1936). Since the beginning of the 21st century stress became an important area of multidisciplinary research and many mechanisms and components of stress have been described.

Stress affects almost all functional systems of the organism including the brain through cascades of hormonal reactions and activation of the autonomic nervous system.

Psychological challenges that we are experiencing on a daily basis are among the most powerful stimuli to induce neuroendocrine, autonomic and behavioural changes (Kovacs et al., 2005). These in turn could facilitate the onset and progression of many diseases. Variability in the stress response depends on the genetic and epigenetic background of each individual (DeRijk et al., 2010). Since 1914 an abundance of data suggested that stress can induce lasting molecular and physiological changes in the brain and its output, behaviour. The brain represents a central regulator which controls the behavioural and physiological responses to stressful events. In turn, brain function is controlled by positive or negative feedback mechanisms (McEwen, 2007). Moreover, chronic stress affects the expression level of genes, including brain-derived neurotrophic factor, cAMP-response-element binding protein, serotonin receptors and components of the hypothalamic-pituitary-adrenal (HPA) axis in experimental models (Alfonso et al., 2005), thus contributing to lasting changes in physiology and behaviour.

At the same time an important part of gene expression regulation is orchestrated by epigenetic components, such as DNA methylation, histone modification, chromosome remodeling, and small non-coding RNAs (microRNA, miRNA). Chronic stress may be an important contributing factor not only to genetic, but also to epigenetic changes in the brain. The understanding of the interaction between genetic and epigenetic components in the brain under a stressful condition can provide an insight into pathogenic processes that contribute to neurological diseases. For example, recent studies have shown that chronic stress is an important factor in the development of hypertension, gastrointestinal

disorders, immune suppression, reproductive dysfunction and mental disorders (McEwen, 2004, Sandi and Pinelo-Nava, 2007).

The chronic restraint stress paradigm in rodents is a model to study the effects of psychological stress. Chronic restraint stress causes different changes in the organism on the behavioural, physiological, and molecular levels. Stress-induced changes in the brain involve the hypothalamus, hippocampus, striatum, pituitary, pineal gland, amygdala, and spinal cord.

Preliminary data suggest that twenty minutes of daily restraint stress during two weeks causes behavioural impairments in motor function in rats (Jadavji and Metz, 2008). Observed behaviour impairments might be caused by changes in the brain areas that regulate motor functions, such as motor cortex and cerebellum. The role of motor cortex in motor function is well studied, while functions of the cerebellum in motor behavioural impairments remain to be investigated. To our knowledge, there are no data on mRNA and miRNA expression in the cerebellum after restraint treatment. We hypothesized that alteration in animal behaviour after chronic restraint stress is due to different cerebellum-specific changes in miRNA and protein encoding gene expression.

The major purpose of this research was to quantify the extent of epigenetic adaptation to a chronic mild stressor in the cerebellum using a rat model. mRNA and miRNA expression profiles were assayed in animals exposed to two weeks of daily restraint stress and two weeks after cessation of stress in comparison to no-stress controls.

The results revealed that expression of 39 genes and three miRNAs was changed after two weeks of restraint treatment. Furthermore we verified one putative target for one of the changed miRNAs and expression of four randomly selected genes. These data suggest that even a very mild stressor can cause molecular changes in the brain, which might play an important role in the onset and progression of neurological diseases.

2. LITERATURE REVIEW

2.1. Physiological, molecular, and behavioural correlates of stress

Different components of the central nervous system (hypothalamus, pituitary, brain stem) and peripheral components (efferent limbs of sympathetic nervous system, adrenal medulla and adrenal cortex) become active in response to an internal or an external stressor (Weinstock, 2010). It was shown that stress and stress hormones could alter neurogenesis in the adult mammalian brain (Gould et al., 1997, Kirby and Kaufer, 2010). According to Selye, adrenocorticotrophic hormone (ACTH) is released from the pituitary gland in response to stress, followed by subsequent secretion of glucocorticoids from the adrenal glands. Abundant research was done since that time which showed that function and regulation of the HPA axis is much more complex and involves the brain on many levels. The paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) that activate the pituitary gland, which in turn, releases ACTH thus stimulating the adrenal glands. In response to activation the adrenal glands release glucocorticoids into the bloodstream. Aside from the brain's prominent feedback mechanisms in response to circulating glucocorticoids, central effects also involve changes in gene expression, protein synthesis and their storage, emphasizing a crucial role of the brain in the temporary and permanent consequences of stress (Levine, 2005). Thus, glucocorticoids through their respective receptors in the brain, the mineralocorticoid and glucocorticoid receptors, are able to participate in multifarious genomic and nongenomic responses throughout the organism (Welberg and Seckl, 2001, Herman et al., 2003, Morris, 2007); see reviews by (de Kloet et al., 2005, McEwen, 2007, McEwen and Gianaros, 2010).

The effects of the stress response can be harmful or beneficial, depending on the type and intensity of stress, its duration, individual differences in coping with stress, duration and timing of stress hormone exposure (Sapolsky et al., 2000, Joels et al., 2007). The main responsibility for the outcome of the stress response on a hormonal level is given to glucocorticoids (cortisol in primates and corticosterone in most rodents, hereafter abbreviated as CORT) (Sapolsky, 1999, Sorrells et al., 2009). CORT plays a protective role by preventing the stress response system from overshooting (Munck et al., 1984, Sapolsky et al., 2000). Excessive amounts of CORT are beneficial or harmless for a few days, but can lead to fatal consequences if prolonged (Sapolsky et al., 2000). Dysregulation in the action of glucocorticoids was implicated in increased susceptibility to many stress-related brain diseases and other pathological conditions (de Kloet et al., 2005, de Kloet et al., 2009, Oitzl et al., 2010).

Stress impairs the body's defenses against agents such as bacteria, viruses, and toxins, thus making the body more susceptible to disease (Sapolsky, 2002). Psychological types of stress modulate stress physiology and can trigger a stress response in the absence of physical damage and homeostatic imbalance (Sapolsky, 2002). According to Sapolsky, the stress response is ideal for coping with short-term physical stressors. The system did not evolve, however, to deal with chronic activation, and therefore a long-term stress response, which is usually not activated for a physiological reason, may eventually result in stress-related disease (Sapolsky, 2000b, 2002).

The stress response can be defined as a physiological and behavioural adaptation to the emotional or physical threats, either actual or imagined (de Kloet et al., 2009). Stress and stress hormones are known to cause impairments in working memory function in both humans (Kirschbaum et al., 1996, de Kloet et al., 1999, McEwen, 2000, Lupien et al., 2005) and animals (Diamond and Park, 2000, Cerqueira et al., 2007), enhance or impair learning, memory consolidation and memory retrieval (Overmier and Seligman, 1967, Faraji et al., 2009). Other behavioural changes caused by stress in rodents include inhibition of exploratory activity (Weiss et al., 1980) and impaired gross and skilled motor function (Jadavji and Metz, 2008). Nevertheless, behavioural changes in stressed animals may also occur in the absence of elevated CORT levels suggesting that other mechanisms, such as emotional responses to a stressor, may modulate behaviour (Metz et al., 2005).

Furthermore, chronic exposure to stress can lead to a suppression of cell proliferation (Czeh et al., 2002, Pham et al., 2003), and hippocampal atrophy (Watanabe et al., 1992, Magarinos and McEwen, 1995b, Magarinos et al., 1996, Sapolsky, 2000a). Accordingly, stress also affects recovery from brain damage, such as diminishing compensation in a rat model of stroke (Kirkland et al., 2008) and exaggerating neural loss in a rat analogue of Parkinson's disease (Smith et al., 2008).

Changes in behaviour and recovery from brain damage are related to changes on the molecular level. These include changes in gene expression, followed by altered protein and metabolite levels (Coirini et al., 1985). Exposure to chronic restraint stress in

rodent models, for instance, leads to changes in gene expression in different brain areas including frontal cortex (Lee et al., 2006), prefrontal cortex (Chen et al., 2008), hypothalamus (Harris et al., 2006, Lee et al., 2006, Chen et al., 2008), hippocampus (Zuena et al., 2008), pineal gland (Dagnino-Subiabre et al., 2006), and the amygdala (Kim and Han, 2006). Changes in gene expression as well as RNA levels are regulated by many parameters including epigenetic mechanisms. These involve changes in the methylation pattern, histone modifications, altered chromatin structure and production of miRNA. Recent studies showed a strong correlation between DNA methylation and behaviour (Colvis et al., 2005, Champagne et al., 2006).

In this chapter we discussed physiological, molecular, and behavioural correlates of stress. The next chapter covers the role of stress in early-life experiences.

2.2. Early-life stress versus late-life influences

During the life-span some periods appear to be more critical than others in terms of sensitivity to stress. A particularly stress-sensitive period is the perinatal period (in humans, it is an interval approximately from the 28th week of gestation to the 28th day after birth). Animal models indicate that events during pregnancy and early-life mother-infant interactions can permanently alter the stress-regulating systems in the offspring (Oitzl et al., 2010). Recent research has demonstrated that long-lasting impact of adverse experience early in development on behaviour later in life results from long-term changes in gene expression mediated by epigenetic components (Shepard et al., 2009).

Among other sensitive developmental periods which might play an important role in the establishment of epigenetic marks is the slow growth period (SGP) before the prepubertal peak in growth velocity. Human epidemiological data shows that different food availability during the SGP alters the transgenerational response, such as longevity and incidence of cardiovascular disease and diabetes (Kaati et al., 2002, Kaati et al., 2007).

The social environment and endocrine experiences early in life might lastingly influence brain function and modulate the adult behavioural phenotype (Crews, 2008). Early-life experiences and genetic background are important factors that can cause an imbalance in stress-regulating systems, which lead to establishing a phenotype vulnerable to later-life stressors (Oitzl et al., 2010). Maternal care influences gene expression, behavioural and HPA axis responses in the offspring, thus predetermining health outcome

in the adult offspring possibly through various generations (Meaney et al., 2007, Weaver, 2009). Work in rats has revealed that the effects of maternal care are associated with epigenetic regulation of glucocorticoid receptor expression in the brain (Weaver et al., 2005, Weaver et al., 2007). Early-life stress was shown to cause memory alterations in mice and was accompanied by an increase in AVP expression, which, in turn, was associated with sustained DNA hypomethylation of multiple CpG residues of the Avp enhancer region (Murgatroyd et al., 2009).

In this chapter we discussed the impact of early-life experience on health and disease. In the next chapter we will focus on the role of stress in neurological disease.

2.3. Stress as an important contributing factor to neurological disease

An important concept of stress physiology is that turning on the stress response for too long is likely to lead to the development of a chronic disease (Sapolsky, 2000b) (Fig. 2.1).

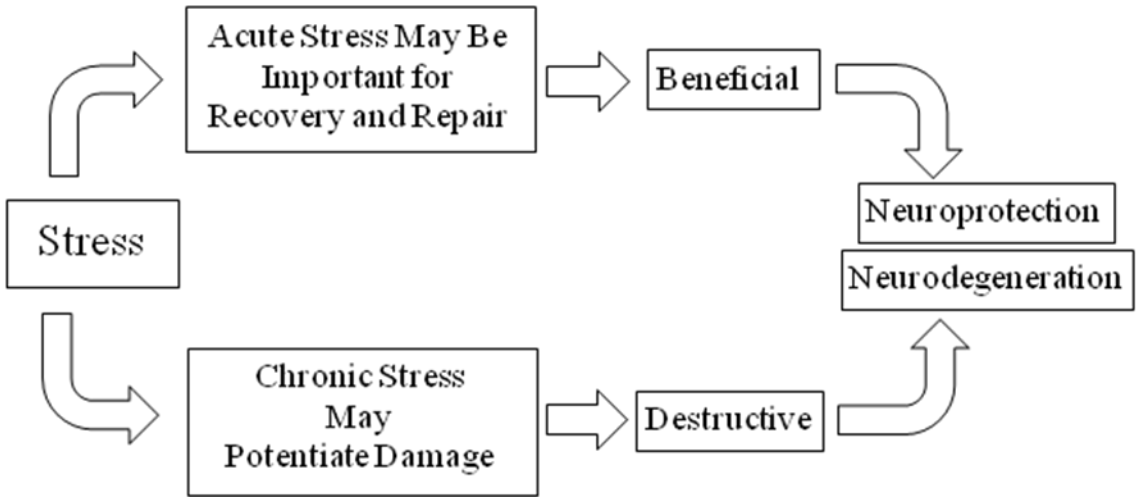


Figure 2.1. Schematic representation of stress outcomes.

The factors which are involved in the onset and development of neurodegenerative disorders are begun to be identified, but the mechanisms of pathogenesis still remain unclear. Nevertheless, different neurological disorders share common risk factors, which may lead to synaptic dysfunction, neuronal degeneration and cell death, thus contributing to the disease. These common factors include lack of physical activity, lack of dietary folate, oxidative stress associated with aging, metabolic impairment, dysregulation of ion homeostasis, DNA damage, apoptosis, and excitotoxicity (Mattson, 2003). On the other hand, these factors could cause alterations in epigenetic regulation in the organism, thus they might impact onset and development of various neurodegenerative diseases through mechanisms of epigenetic response. Another important factor which might play a key role in the epigenetic response and the

development of pathological processes in the brain is stress, which together with other agents may lead to the onset and progression of Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS) or development of stroke (Fig. 2.2).

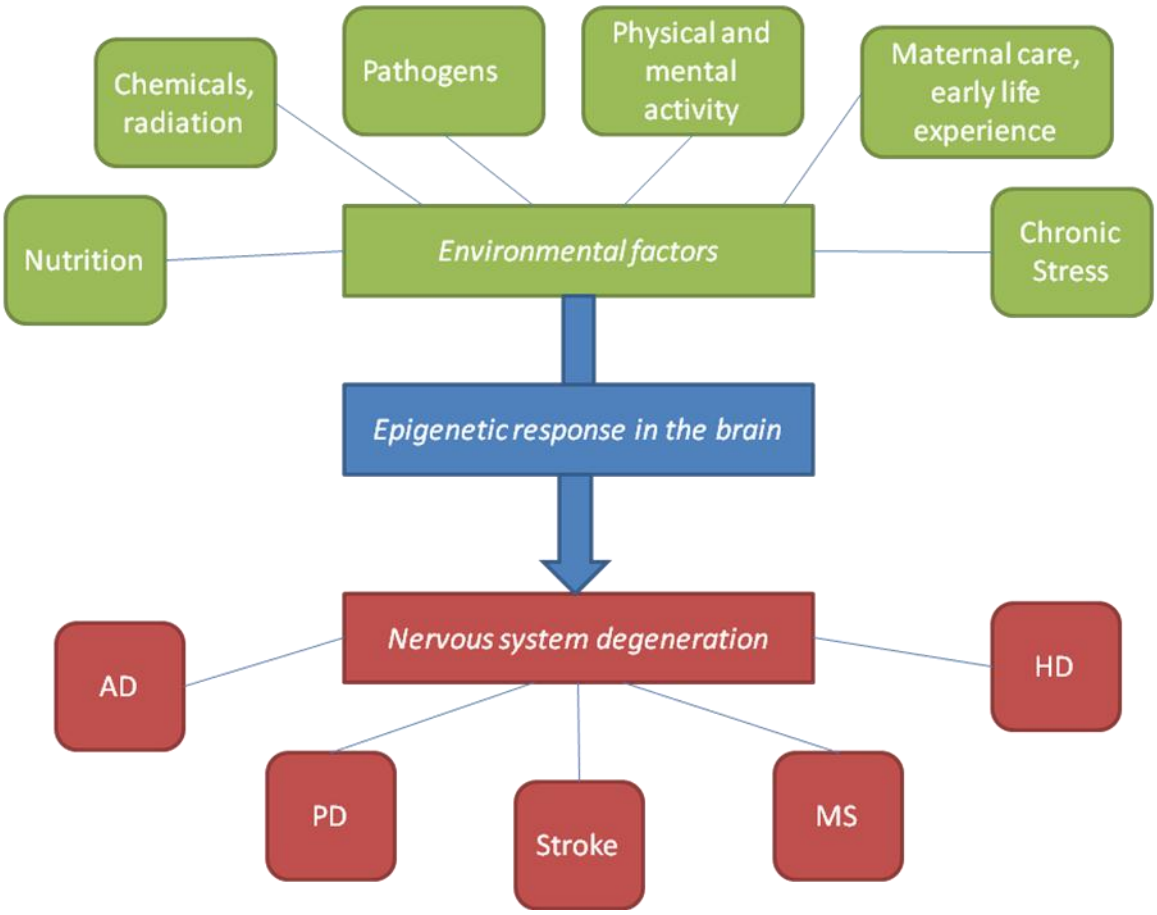


Figure 2.2. Role of environmental factors in the epigenetic response in the brain and neurological diseases. Different environmental factors such as nutrition, physical and mental activity, maternal care and early life experience, exposure to chronic stress, chemicals and radiation, and pathogens can alter the epigenetic pattern in the brain. These processes in turn may promote neuronal degeneration and contribute to the onset and development of different neurological conditions, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), and stroke.

2.4. Epigenetics in neurobiology

The completion of the Human Genome Project in 2003 was a hallmark event in biology, however many mysteries in such a complex biological process as gene expression regulation remain still unrevealed. The rise of a new discipline of epigenetics in recent decades promises to answer some of these questions. The understanding of how the environment influences genetics evolves through centuries, from Lamarck and Darwin till nowadays, after the term epigenetics was coined by Conrad Waddington in the 1940s. Epigenetics can be defined as the study of heritable changes in gene expression that are not caused by changes in the nucleotide sequence of the DNA. Modifications in the epigenome play a vital role in many biological processes and molecular mechanisms in the organism. The main components of epigenetic regulation are DNA methylation, histone modification, chromatin remodeling, and non-coding RNAs. The recognition of the role of epigenetics in human disease started in oncology, but now it is extended to many other disciplines (Urdinguio et al., 2009). Recent studies suggest that epigenetic mechanisms might play an important role in the mediation of enduring changes in brain function (Darnaudery and Maccari, 2008). Research in the last decades highlighted the role of epigenetic regulation in many processes in the nervous system, such as neuron development and function, neuronal plasticity, and memory formation, raising the question of a possible role of epigenetics in neurological disease etiology. Current research is focused on the role of epigenetic components in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, suggesting its possible role in neuroprotection.

2.4.1. DNA methylation

DNA methylation is part of the covalent structure of the DNA and in eukaryotes it consists of the covalent addition of a methyl group at the 5'-position of cytosines (Razin and Riggs, 1980). In mammals, DNA methylation occurs mostly at cytosine-phosphate-guanine sites (CpG sites) and is associated with untranscribed chromatin and gene silencing, whereas active chromatin is generally associated with hypomethylated DNA (Kornberg, 1974, Razin and Riggs, 1980). Other functions of DNA methylation include regulation of genome stability through silencing repetitive elements, role in chromatin structure, genomic imprinting, inactivation of the X chromosome, and protection of DNA from degradation by nucleases. DNA methylation patterns carry the information about the regulation of gene expression and could be transmitted to the next generation. First molecular model for heritability of gene activation during development by DNA methylation was proposed by Robin Holliday and colleagues (Holliday and Pugh, 1975, Crews, 2008).

The process of establishing and maintaining a DNA methylation pattern is complex and involves the interaction between methyltransferases and methyl-CpG binding proteins (MBDs). MBDs are the proteins that read and interpret the information of methylation patterns. For many years it was thought that DNA methylation is extremely stable and irreversible and that changes in DNA methylation patterns might occur only during critical periods in embryogenesis (Razin and Riggs, 1980). The new data suggest that DNA methylation is dynamic (Bhattacharya et al., 1999, Weaver et al., 2004, Levenson et al., 2006, Miller and Sweatt, 2007). DNA methylation patterns are

actively maintained by DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine onto the 5' position of the cytosine ring (Adams et al., 1979, Van Emburgh and Robertson, 2008). Demethylation events may occur in the absence of dNTPs (Szyf et al., 2008), but their exact mechanisms still need to be determined (Reik, 2007). According to the Szyf group DNA methylation patterns represent a balance of methylation and demethylation reactions, which provide a platform for gene-environment interaction (Ramchandani et al., 1999).

2.4.2. DNA methylation in neurobiology

During brain development DNA methylation is believed to be important in regulating the proliferation of neural stem cells and their differentiation into neurons and glial cells (Mattson, 2003). Methylation of DNA in the brain is catalyzed by three main enzymes, DNMT1, DNMT3a, and DNMT3b. DNMTs are expressed throughout neural development and promote neuronal survival and plasticity, thus influencing stress responses in the brain (Klose and Bird, 2006, Ooi et al., 2007, Mehler, 2008). MBDs were shown to play an important role in brain development and cognitive functions such as learning and memory (Feng et al., 2007, Chahrour et al., 2008, Mehler, 2008).

A growing body of research extends the function of DNA methylation beyond the developing nervous system. DNA methylation was shown to be important in neuronal apoptosis in the process of brain maturation (Yakovlev et al., 2010). Feng et al. (2010) showed that Dnmt1 and Dnmt3a are required for synaptic plasticity, learning and memory in adult CNS neurons (Feng et al., 2010). Recent data suggest an involvement of

DNA methylation processes in CNS repair (Iskandar et al., 2010). Iskandar and colleagues have shown that axonal regeneration after spinal cord and sciatic nerve injury relies on the integrity of DNA methylation pathways (Iskandar et al., (2010). Their study demonstrated a correlation between regeneration after injury, spinal cord DNA methylation and the expression of the *de novo* DNA methyltransferase enzymes Dnmt3a and Dnmt3b, but not Dnmt1. Research in the last decades has shown that DNA methylation patterns are altered in various pathological conditions of the nervous system (McGowan et al., 2008b, Feng and Fan, 2009).

2.4.3. Chromatin remodeling and histone modification

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, and ubiquitination. The first histone modification - lysine methylation - was reported by Murray in 1964 (Murray, 1964). This was followed by the discovery of other histone modifications, such as serine phosphorylation (Kleinsmith et al., 1966), lysine acetylation (Gershey et al., 1968, Vidali et al., 1968), ubiquitination and ADP-ribosylation (Goldknopf and Busch, 1975, Ueda et al., 1975)., 1975), and SUMOylation (Shiio and Eisenman, 2003) (for more details see (Sims et al., 2008)).

The basic structural subunit of chromatin is the nucleosome, which consists of DNA, wrapped around an octamer, and formed by the histone proteins, termed H1, H2A,

H2B, H3 and H4 (Kornberg, 1974). Histone modifications are catalyzed by histone modification enzymes, including histone acetyltransferases (HAT), histone deacetylases (HDAC), histone methyltransferases (HMT) and histone demethylases. A change in a "histone code", or specific pattern of histone modifications could result in gene activation or silencing (Jenuwein and Allis, 2001). Despite the above mentioned role in gene silencing, there is a controversy in the literature, whether nucleosome modifications should be considered as epigenetic changes, since they lack the fundamental epigenetic property of self-propagation (Ptashne, 2007).

2.4.4. Chromatin remodeling and histone modification in neurobiology

Understanding the concept of chromatin remodeling may provide an insight of how stable changes in gene expression in the brain produce long-lasting changes in behaviour (Colvis et al., 2005). Increasing evidence indicates that changes in the chromatin structure occur not only during development, but also in mature neurons (Tsankova et al., 2007, Renthal and Nestler, 2008). Neuronal signaling appears to be strongly regulated by addition and removal of histone acetylation, histone and DNA methylation (Renthal and Nestler, 2008). Recent studies showed that neuronal levels of monoacetylated H4 decrease progressively during aging, whereas levels of acetylation of non-histone proteins may increase in neurons during development and with age (Mattson, 2003).

It has been shown that chromatin remodeling via post-translational modification of histone proteins, primarily histone H3 phosphorylation and acetylation, is important in

the formation of long-term memory (Alarcon et al., 2004, Korzus et al., 2004, Levenson et al., 2004, Gupta et al., 2010, Peleg et al., 2010). Some data suggest that histone methylation is actively regulated in the hippocampus and facilitates long-term memory formation, while deregulation of H4K12 acetylation may cause memory impairments in the aging mouse brain (Gupta et al., 2010, Peleg et al., 2010). Interestingly, it was shown recently that mammalian neurons have a specialized chromatin remodeling enzyme, histone lysine specific demethylase 1, suggesting its role in gene repression (Zibetti et al., 2010).

HDACs catalyze the removal of an acetyl group from the N-terminal tails of histone proteins, while histone acetyl transferases (HATs) catalyze the reverse reaction (Sleiman et al., 2009). The alteration in balance between HDAC and HAT functions can lead to cancer as well as to neurodegenerative disease (Hahnen et al., 2008). HDAC inhibitors have shown therapeutic efficacy in animal models of neurodegenerative diseases, including HD, PD, AD (Fischer et al., 2007, Abel and Zukin, 2008), suggesting a neuroprotective role. HDAC inhibitors are currently available for therapy for several neurological disorders. A protective role of HDACs was also implicated in a rodent stroke model (Faraco et al., 2006). Faraco and colleagues demonstrated that HDACs promotes expression of neuroprotective proteins within the damaged brain area (Faraco et al., 2006).

2.4.5. Non-coding RNAs

Around 98% of transcribed genomic DNA are non-coding RNAs that do not function as messenger, transfer or ribosomal RNAs (Yu, 2008). miRNAs are small non-

coding single-stranded RNA molecules approximately 22 nucleotides long, which can regulate gene expression via an RNA interference pathway by altering mRNA stability and translational initiation. Each miRNA can regulate thousands of different mRNA targets (Nelson et al., 2008).

miRNAs were first described in *Caenorhabditis elegans* (Lee et al., 1993, Wightman et al., 1993). Further research reported their involvement in many biological processes, such as cellular development, differentiation, proliferation, cell division, apoptosis, onset and progression of cancer and other diseases. The expression of miRNA molecules depends on different factors and can be regulated by other epigenetic agents, such as DNA methylation and chromatin structure (Saito and Jones, 2006).

2.4.6. RNA-mediated gene silencing

RNA-mediated gene silencing is an evolutionarily conserved process in which dsRNA molecules interfere with target genes and initiate a cascade of events that repress expression of target genes in a sequence-specific manner (Rodriguez-Lebron and Paulson, 2006). In animals, the double-stranded RNA-specific endonuclease Dicer produces two classes of small RNAs: miRNAs and small interfering RNAs (siRNAs). miRNAs function through attenuation of mRNA translation, whereas siRNAs direct RNA destruction via the RNA interference (RNAi) pathway (Hutvagner and Zamore, 2002). In order to inhibit translation, nucleotides 2-7 of the 5’ end of the guide strand of miRNA (seed sequence) must be of a perfect or near-perfect complementarity to the target sequence in the mRNA 3’UTR (Smalheiser and Torvik, 2006).

2.4.7. Non-coding RNAs in neurobiology

The expression of miRNAs is cell- and tissue-specific and miRNAs are particularly abundant in the brain (Kosik, 2006). Many miRNAs are expressed in a spatially and temporally controlled manner in the nervous system, suggesting that their regulation may be important in neural development and function (Barbato et al., 2009). Recent research showed that miRNAs play important roles in nervous system development and function, neurodegenerative and neuropsychiatric diseases as well as in brain cancer (Mehler and Mattick, 2006).

Evidence in the past years has indicated that miRNAs may be a contributing factor to aging-related neurodegenerative diseases (Colvis et al., 2005, Nelson and Keller, 2007, Nelson et al., 2008). Schaefer et al. (2007) for instance demonstrated that substantial loss of mature miRNA in the cerebellum of mice with knocked-out Dicer gene causes progressive neurodegeneration in a mouse model, suggesting that the same mechanism may be relevant in human neurodegenerative disorders (Schaefer et al., 2007).

2.5. Role of epigenetic regulation in stress response and its influence on the development of neurological diseases

2.5.1. Epigenetics of the stress response

Resent research on epigenetic regulation of the stress response is mainly focused on the role of glucocorticoids. Growing evidence suggests that mineralocorticoid (MR) and glucocorticoid (GR) signaling in the brain might be regulated by epigenetic components (de Kloet et al., 2009). For instance, Uchida et al. (2008) compared the stress response in the Fisher 344 rat strain (which represent a stress-hyperresponsive strain) and the Sprague-Dawley strain. They reported that after two weeks of restraint stress Fisher 344 rats had lower expression of GR protein but not mRNA in the hypothalamus. Authors also demonstrated that miR-18a was overexpressed in the hypothalamus in Fisher 344 rats as compared to Sprague-Dawley rats, and that miR-18a inhibits translation of GR mRNA *in vitro.* Their findings suggest that miR-18a regulation of GR translation could play a role in susceptibility of stress-related diseases (Uchida et al., 2008). On the other hand, Vreugdenhil et al. (2009) demonstrated that miR-18 expression in the brain is 8-fold lower than what is needed *in vitro* to regulate the expression of GR protein, thus the role of miR-18 in the brain still needs to be investigated (de Kloet et al., 2009, Vreugdenhil et al., 2009).

Epigenetic effects of glucocorticoids, mainly through their role in early life experience, were well demonstrated by the Meaney and Szyf groups. They suggested that variations in maternal care might be responsible for the individual differences in stress reactivity across generations on a non-genetic basis (Francis et al., 1999). In particular, it was shown in a rodent model that the offspring of mothers that exhibit high level of pup

licking/grooming (LG) behaviour show an increased level of GR mRNA expression in the brain. The authors proposed that this mechanism is regulated by DNA methylation (Weaver et al., 2007). The Meaney and Szyf groups demonstrated that increased LG behaviour is associated with demethylation of the 5' CpG site within the exon 1_7 of GR gene promoter region (Weaver et al., 2004). Moreover, they demonstrated that dietary methionine treatment reverses the stress response in the adult offspring of high LG mothers (Weaver et al., 2005).

2.5.2. Neurological disease background

Neurological diseases are disorders primarily affecting the nervous system. There are more than 600 nervous system disorders including stroke, epilepsy, head and spinal cord injury, Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease and brain tumors. Here we will focus on some of the most known and best studied diseases.

Ischemic stroke commonly results from occlusion of a major artery in the brain, which causes cell death in the affected area (Sims and Muyderman, 2010).

Alzheimer's disease (AD) is a common form of dementia, which is characterized by cognitive, and in the advanced stage, by physical impairments. AD is a slowly progressive complex disease of the brain, the development and progression of which depends on many factors including genetic and environmental components. Although the formation of neuritic plaques and neurofibrillary tangles in the brain are associated with

AD (Tiraboschi et al., 2004), the causes of onset and progression of this disease remain poorly understood.

Parkinson's disease (PD) is a progressive neurological disorder characterized by the selective and progressive loss of dopaminergic neurons in the midbrain substantia nigra, which causes a large number of motor and non-motor impairments (Jankovic, 2008, Sleiman et al., 2009). Near six million people worldwide have PD, and it affects 1% of the population over 65 (Gasser, 2001, Abel and Zukin, 2008).

Multiple sclerosis (MS) is an inflammatory autoimmune disorder, characterized by damage of myelin and axons in the brain and spinal cord (Compston and Coles, 2002, 2008).

Huntington's disease **(**HD) is an autosomal, dominant, neurodegenerative disease, which affects about 3 in 100 000 individuals (Barbato et al., 2009). HD is characterized by incoordination, progressive involuntary movements, neuropsychiatric disturbancest, and cognitive decline (Montoya et al., 2006, Walker, 2007). HD is caused by a polyglutamine expansion in the 5′-coding region of the huntingtin (*htt)* gene (Bates, 2001).

The molecular mechanisms underlying the above mentioned disease onset and progression remain poorly understood (Sleiman et al., 2009), which leads to the absence

of effective cures. Therefore, up to date the above mentioned diseases have no known cure.

In recent years a growing body of evidence suggests involvement of an epigenetic component, particularly an exciting therapeutic potential of HDAC inhibitors, which have shown broad efficiency in cellular and animal models of many diseases, including stroke, AD, PD, and HD (Ratan, 2009, Sleiman et al., 2009).

2.5.3. Epigenetic mechanisms underlying neurodegenerative diseases

Neurodegenerative diseases are diseases in which the nervous system progressively and irreversibly deteriorates. Recent studies suggest that neurodegenerative diseases are mediated by aberrant epigenetic mechanisms. Genes that are responsible for the inherited form of neurodegenerative disorders, encoding alfa-synuclein and Parkin in PD, presenilin-1, presenilin-2, Tau in AD and huntingtin in HD represent potential targets for epigenetic regulation. Genes encoding other proteins, involved in a complex cascade of chemical reactions, leading to the process of neurodegeneration still need to be identified.

2.5.4. miRNA and neurodegenerative diseases

The discovery of an association between miRNA deregulation and different neurological disorders opened a new area of research on neurodegenerative illnesses. miRNAs have been implicated in several neurologic disorders, including AD, PD, HD, and MS (Barbato et al., 2008, Maes et al., 2009, Otaegui et al., 2009).

Alteration in miRNA function and aberrant miRNA expression contributes to human disease and has a potential as disease biomarkers (Chang and Mendell, 2007, Maes et al., 2009). miRNA profiling in human patients with AD identified a number of miRNA up-regulated in peripheral blood mononuclear cells (Schipper et al., 2007), however, further investigations still need to be done to assess their potential as blood-based diagnostic and prognostic biomarkers.

Noncoding RNAs are also necessary for survival of neurons in PD and other neurological disorders (Hebert and De Strooper, 2007). Systemic increase in specific miRNAs may suppress various cellular functions in AD (Maes et al., 2009). miRNAs were proposed to be important in the molecular mechanisms implicated in MS, suggesting their role as candidate biomarker targets in MS (Otaegui et al., 2009). Work of Otaegui et al. (2009) suggests that hsa-miR-18b and hsa-miR-599 may be involved at the time of relapse, while hsa-miR-96 may play a role in remission (Otaegui et al., 2009).

Various miRNAs were reported to be altered in rodent models of AD (Table 2.1). Considering the fact that some miRNAs are highly conserved between mammals, while other are poorly conserved, additional research needs to be done in this area to determine whether all of these miRNAs are active in the human brain and have similar functions.

Table 2.1. miRNAs associated with neurodegenerative disorders.

miRNA	Brain area	Status	Disease	The role in disease	References
miR-9	hippocampus	↑Up-regulated	AD in animal model	Needs to be investigated	(Lukiw, 2007)
miR-128					Kim et al., 2007
miR-15a	Cortex	↓Down-regulated		Candidate binding sites in the 3'UTRs of beta site amyloid precursor protein (APP) cleaving enzyme (BACE)	Barbato et al., 2009
miR-29b-1					(Hebert et al., 2008)
miR-19b					
miR-9	Cortex	↓Down-regulated		Candidate binding sites in the 3'UTRs of BACE and PSEN1	Barbato et al., 2009
101	Cortex	↓Down-regulated		Candidate binding sites in the 3'UTR of APP	Barbato et al., 2009
15a					
106b					
Let-7					
miR-210	Cortex	↓Down-regulated		Seem to be unrelated to obvious targets	Barbato et al., 2009
miR-181c					
miR-22					
miR-26b					
miR-363					
miR-93					
miR-9	Cerebrospinal fluid	↓Down-regulation		Correlation to impaired neurogenesis and neuronal differentiation	Barbato et al., 2009
miR-132					Maes et al., 2009
miR-423	hippocampus	↑Up-regulated		Modulate IDH2 (isocitrate dehydrogenase 2) expression and IDH2 reduction is involved in oxidative stress in AD prefrontal cortex	Barbato et al., 2009
miR-98	cerebellum	↓Down-regulation			
miR-107	Medial frontal cortex	↓Down-regulation		Correlates with BACE1 mRNA increase, miR-107 modulates expression of BACE1 in cell culture	(Wang et al., 2008)
	Motor cortex				
miR-34a	Peripheral blood mononuclear cells	↑Up-regulation	AD in human patients	May repress neuronal and synapse function	(Wang et al., 2008)
miR-181b				May repress DNA repair and stress response	Maes et al., 2009
miR-520h				May play role in immune response, signal transduction	
miR-371				May repress mitochondria function	

miR-517*				Could influence cell survival	
let-7f				May repress immune and injury responses	
miR-200a				Might be important in stress response	
miR-133b	midbrain	↓Down-regulated	PD in human patients	Differentiation and function of dopaminergic neurons	(Kim et al., 2007)
miR-433				Candidate binding site in the 3'UTR of FGF 20, modulate expression of alfa-syneclein, which might cause greater susceptibility to PD	(Wang et al., 2008)

2.5.5. Role of histone modification and chromatin remodeling in neurological diseases

HDACs are considered to play a protective role in the brain (Jiang et al., 2008). Evidence from the past years has indicated that the balance between HATs and HDACs is impaired in many neurodegenerative conditions (Saha and Pahan, 2006). HDAC inhibitors are very promising for the therapy of neurodegenerative diseases (Fischer et al., 2007). They have shown therapeutic efficacy in animal models of HD (Ferrante et al., 2003) and stroke (Ren et al., 2004, Faraco et al., 2006). HDAC inhibitors have shown a protective effect against oxygen and glucose deprivation and against oxidative-stress-induced cell death *in vitro*, which strongly suggests their potential in treatment for stroke patients (Sleiman et al., 2009). For a broader discussion of the role of HDAC inhibition in neurodegenerative diseases see Sleiman et al. (2009).

2.5.6. Role of methylation in neurological diseases

Evidence from the study of monozygotic twins suggests that epigenetic rather than genetic factors might be responsible for differences in DNA methylation patterns in adult tissues (Fraga et al., 2005). Alteration in DNA methylation during embryogenesis and possibly during adult life can lead to stable changes in gene expression and development of different pathological conditions (Feinberg, 2007, McGowan et al., 2008a).

Several approaches are available to assess DNA methylation patterns in normal and disease-associated cells and tissues, but for neurological diseases, the few DNA methylation markers that have been identified so far require further confirmation (Urdinguio et al., 2009). Studies of cultured neurons have shown that alterations in methylation and acetylation can cause cell death, thus suggesting their role in the cascade of reactions leading to neurodegeneration (Mattson, 2003).

Altered DNA methylation patterns were observed in AD, PD, and MS (Urdinguio et al., 2009). A high-throughput screening study in human cerebral cortex revealed two genes which are differently methylated in Alzheimer's tissues, as compared to controls: *S100a2* (S100 calcium binding protein A2) and *Sorbs3* (sorbin and SH3 domain containing the 3 cell-adhesion protein) (Siegmund et al., 2007). *Sorbs3* was hypermethylated, while analysis of *S100a2* revealed a decrease in DNA methylation in Alzheimer patients as compared to control cases. Alteration in the methylation pattern of *S100a2* and *Sorbs3* might play a role in synaptic impairment in AD (Urdinguio et al., 2009). Also, hypermethylation of the promoter region of *Nep* (neprilysin) gene in murine

cerebral endothelial cells was reported, suggesting its role in amyloid-beta accumulation in AD (Chen et al., 2009).

Recent evidence suggests that alterations in DNA methylation might play a role in the mechanism of MS pathogenesis (Mastronardi et al., 2007). It was shown that DNA methylation of cytosine of the *Pad2* promoter in the white matter from MS patients was one-third of the methylation level in normal brain tissue (Mastronardi et al., 2007). Findings by Mastronardi et al. (2007) demonstrated also 2-fold higher DNA demethylase activity in tissues from MS patients as compared to those of normal, or Alzheimer's, Huntington's and Parkinson's disease white matter. For a more detailed review on epigenetic mechanisms in other neurological diseases see Urdinguio et al. (2009).

3. THE MOLECULAR MECHANISMS UNDERLYING EPIGENETICS OF THE STRESS RESPONSE IN THE CEREBELLUM IN A RAT MODEL

3.1. Materials and methods

3.1.1. Animals

Twenty-four male adult Long-Evans hooded rats, approximately 4 months old (weighing approximately 460 g at the beginning of the study), from Charles River Laboratories International, Inc (Wilmington, MA, US) were used. Animals were habituated to the local environment for a period of three weeks prior to the start of the experiment. The 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 local animal welfare committee.

3.1.2. Experimental groups

Animals were randomly assigned to one of the following experimental groups: Two weeks of daily restraint stress (2WSTRESS, n=6), two weeks naive controls (2WCONTROL, n=6), two weeks of daily restraint stress + two weeks of recovery from stress (4WSTRESS, n=6), four weeks naive controls (4WCONTROL, n=6).

3.1.3. Time course

The 2WSTRESS group was subjected to 14 days of restraint stress, while the 4WSTRESS group received two weeks of recovery after two weeks of restraint treatment. Stressed and respective control animals were sacrificed immediately after the

last day of stress and after 14 days of recovery from stress (Fig. 3.1). Blood samples were collected at baseline, on the first and last day of stress treatment, and on the last day of recovery after stress.

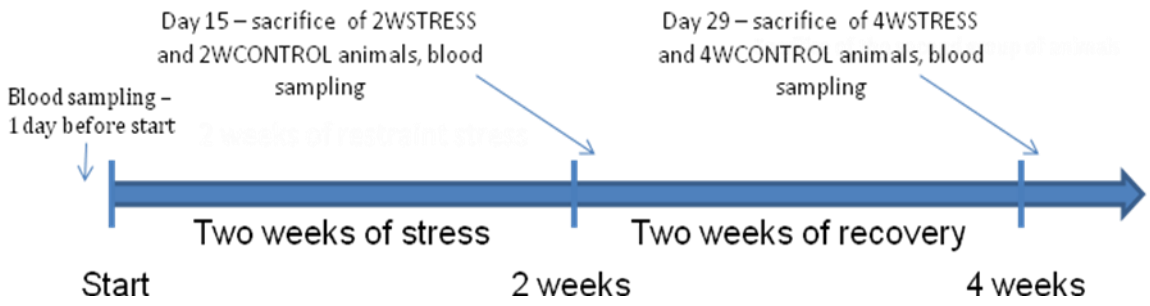


Figure 3.1. Time course of the experimental manipulations.

3.1.4. Physiological manipulations and stress procedure

3.1.4.1. Chronic restraint stress procedure

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. Restraint took place each day at the same time in the morning hours and in a room different from the colony room (Fig. 3.2).

3.1.4.2. Blood sampling

Blood samples were collected between 08:30AM and 11:00AM in order to minimize effects of plasma corticosterone circadian variations (D'Agostino et al., 1982). Samples were collected approximately 30 minutes after initiation of restraint stress (Metz et al., 2005). Rats were anesthetized with 4% isoflurane in 30% oxygen. Syringes and

butterfly catheters were rinsed with heparin prior to use for blood sampling. An average of 0.6 ml of blood was collected from the tail vein using a butterfly catheter. The sample was centrifuged at 4000 rpm for 10 minutes. After centrifugation plasma was collected and stored at -20 ˚C. Plasma corticosterone levels were determined by a corticosterone EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA).

3.1.5. Corticosterone enzyme immunoassay (EIA)

Quantification of corticosterone in plasma samples was performed with the Corticosterone EIA Kit according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). For the assay plasma samples were extracted three times with four volumes of methylene chloride and dried under a stream of nitrogen. The extracts were dissolved in EIA buffer in a volume which is equal to the initial sample volume. In a 96-well plate 100 µl of EIA buffer were added to Non-Specific Binding (NSB) wells, and 50 µl of EIA buffer were added to Maximum Binding (B0) wells. 50 µl of S1-S8 corticosterone EIA standards (5,000, 2,000, 800, 320, 128, 51.2, 20.5, and 8.2 pg/ml, accordingly) were added in duplicates into corresponding wells on the plate. Two dilutions of each sample (50 µl) were added in triplicate into corresponding wells. Then 50 µl of Corticosterone AChE Tracer was added to each well except the Total Activity (TA) and the Blank (Blk) wells, and 50 µl of Corticosterone EIA Antiserum was added to each well except the TA, Blk, and NSB wells. The plate was covered with plastic film and incubated for two hours at room temperature on an orbital shaker. After incubation wells were washed five times with 100 µl of wash buffer. Thereafter 200 µl of Ellman's Reagent were added to each well, and 5 µl of tracer to the TA well. The plate was

covered with plastic film and incubated on an orbital shaker in the dark to allow optimum development. The plate was read at a wavelength of 412 nm. Calculations and data analysis were performed using the original Cayman spreadsheet.

3.1.6. Brain tissue dissection and RNA extraction

The rats were deeply anaesthetized with pentobarbital (WDDC, Edmonton, AB, Canada). After vital signs were discontinued animals were rapidly decapitated. Brains were rapidly removed. The brains of four animals from each group were used for dissection of cerebellum, frontal cortex and hippocampus. The brains of two animals from each group were flash-frozen. Dissected cerebellum was used for DNA/RNA extractions.

3.1.7. DNA/RNA extractions

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

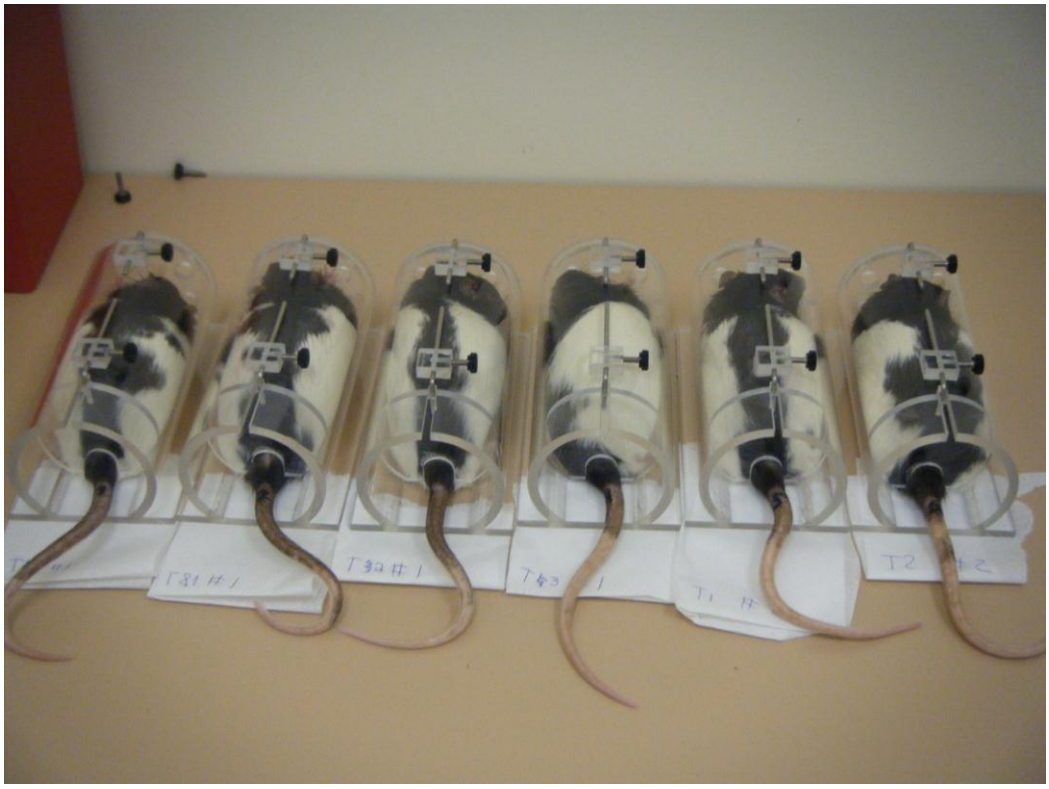


Figure 3.2. Restraint stress procedure. Representative picture of restraint stress treatment. Male Long-Evans rats were placed into transparent plastic tubes for 20 minutes each day.

3.1.8. Plasmid DNA purification

Plasmid DNA was purified from overnight culture (20 ml LB medium supplemented with corresponding antibiotics) using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA).

3.1.9. cDNA synthesis

cDNA synthesis was performed using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas, Barlington, ON, Canada). For the reaction 4.6 µg of total RNA, 1 unit of Ribolock RNase inhibitor, 10 mM dNTPs, oligo(dT_{18}) primers, and

5x reaction buffer were used. The mix was incubated for 1 h at 42˚C and heat inactivated for 5 min at 70˚C.

3.1.10. miRNA microarray expression analysis

Tissue from three animals per group was used for the analysis. Total RNA was extracted from the cerebellum. The miRNA microarray analysis was performed by LC Sciences (Houston, TX, USA; www.lcsciences.com). The assay used from 2 to 5 µg of the total RNA sample, which was fractionated by size using a YM-100 Microcon centrifugal filter (Millipore, Billerica, MA, USA) and the small RNAs (< 300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Two different tags were used for the two RNA samples in dual-sample experiments.

Hybridization was performed overnight on a µParaflo microfluidic chip (LC Sciences, Houston, TX, USA) using a micro-circulation pump. On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to the target miRNA (from miRBase, Welcome Trust Sanger Institute, Cambridge, UK; http://microrna.sanger.ac.uk/sequences/) or control RNA and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 µL 6xSSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C.

After hybridization detection fluorescence labeling using tag-specific Cy3 and Cy5 dyes was performed. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Devices, Sunnyvale, CA, USA) and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD, USA). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter10 (Locally-weighted Regression). For two-color experiments, the ratio of the two sets of detected signals (log_2 transformed, balanced) and p-values of the t-test were calculated; differentially detected signals were those with less than 0.01 p-values (information provided by Jason Mulcahey, Accounts Manager, LC Sciences).

3.1.11. mRNA microarrays

The mRNA microarray analysis was performed by Genome Quebec (Montréal, Quebec, Canada; www. genomequebecplatforms.com). The sample used 250 ng of starting total RNA to hybridize 750 ng of biotin labeled cRNA on the RatRef-12 array. Amplification was performed using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA). Illumina's Gene Expression system uses a "direct hybridization" assay whereby biotin-labeled samples are hybridized individually to an array (Fig. 3.3). Following hybridization the transcripts were detected using Cy3 conjugated streptavidin and scanned. The signal intensities generated provide an indication of the absolute abundance of transcripts within that population. The signal intensities generated from separate arrays can be compared as an indication of the differences between the two sample populations (Illumina, San Diego, CA, USA; www.illumina.com).

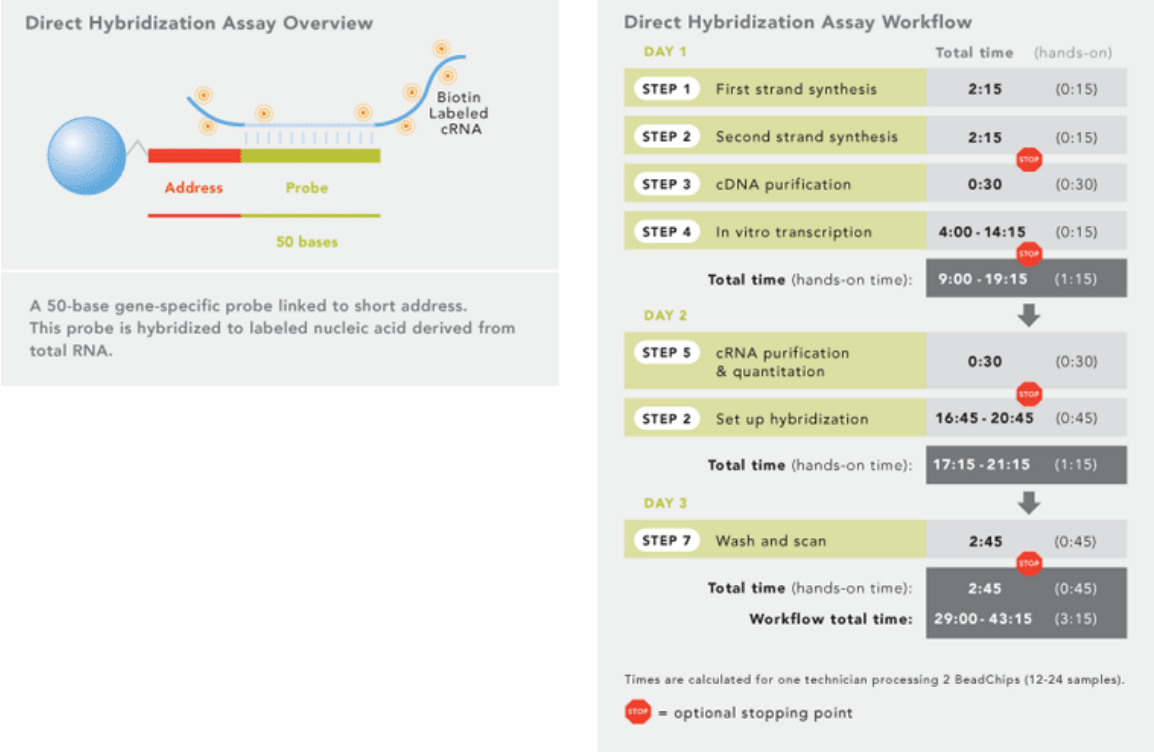


Figure 3.3. Direct Hybridization Assay Overview and Workflow. The Illumina Gene Expression protocol features a first- and second-strand reverse transcription step, followed by a single in vitro transcription (IVT) amplification that incorporates biotin-labeled nucleotides. Subsequent steps include array hybridization, washing, blocking, and streptavadin-Cy3 staining. Fluorescence emission by Cy3 is quantitatively detected for downstream analysis (picture was taken from Illumina's web-site).

3.1.12. Semi-quantitative reverse transcription PCR (sqRT-PCR)

The sqRT-PCR was performed by using Taq DNA Polymerase (Fermentas, Burlington, ON, Canada), specific primers and carried out on an Eppendorf Mastercycler PCR machine (Eppendorf, Hamburg, Germany). Primers were designed using Primer3 v. 0.4.0 software (Rozen and Skaletsky, 2000) and synthesized by Integrated DNA Technologies (San Diego, CA, USA). Each reaction contained 1 µl of cDNA, 0.5 µl of 10 µM forward and reverse primers, 2.5 µl of 10x Taq Buffer, 2.5 µl of 2 mM dNTPs, and 0.25 µl of Taq DNA polymerase (5U/µl) in a total volume 25 µl. PCR conditions and primers' sets are summarized in Table 3.1. PCR conditions where empirically determined

for each set of primers so that the concentration of PCR product was below saturation point. Agarose gel electrophoresis was carried out in 1x TAE buffer using a 1% agarose gel with ethidium bromide. The amplified product was visualized under UV light and quantified using ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ, USA) and normalized to *Gapdh* gene expression.

Table 3.1. Primers and PCR conditions for sq-RT-PCR and cloning.

Gene/ Object	*Primer List name*	*Type of Primer*	*Sequence*	*Note*
Eps15	MS007	for	AAATCTAGATAGTCCCAAAGAAAAGGATCCTGATATG	L=464bp, from *Rattus norvegicus* gDNA or cDNA. For cloning into pGL3 with XbaI and FseI.
Eps15	MS008	rev	AAAGGCCGGCCGGCAAAGCTGTTTAATTAGGGTACTGC	
Nab1	MS013	for	AAATCTAGACTGAGATTCTCCCCCTGCTCTCTGG	L=351bp, from *Rattus norvegicus* gDNA or cDNA. For cloning into pGL3 with XbaI and FseI.
Nab1	MS014	rev	AAAGGCCGGC**C**GGGATCCACGTCTATTAGACTGCAAGG	
EPS15	MS026	for	GCCCCCCCTTCCAAAAGCAGTACCCTAAT	Initial denaturation - 98˚C for 30 sec, 30 cycles with initial denaturation 98˚C for 10 sec, annealing - 62˚C for 30 sec, polymerization - 72˚C for 30 sec, final extention - 72˚C for 3 min.
	MS027	rev	P-AAAAGACACATTTGAAGTTCCCCC	
	MS028	check	GGGGAACTTCAAATGTGTCTTTTGCC	
NAB1	MS044	for	GATTGTCTTCAGCTCCCAGACCA	Initial denaturation - 98˚C for 30 sec, 30 cycles with initial denaturation 98˚C for 10 sec, annealing - 60˚C for 30 sec, polymerization - 72˚C for 30 sec, final extention - 72˚C for 3 min.
	MS045	rev	P-ACACTGGTTATTAAGGCTTAATCTACGAT	
	MS046	check	GTAGATTAAGCCTTAATAACCAGTGTGAT	
ADIPOQ	MS047	for	ATGCTACTGTTGCAAGCGCTCC	Initial denaturation - 95˚C for 3 min, 31 cycles with initial denaturation 95˚C for 30 sec, annealing - 61.6˚C for 30 sec, polymerization - 72˚C for 30 sec.
	MS048	rev	TCAGTTGGTATCATGGTAGAGA	
Ephrinb3	MS049	for	ATGGGGGGCCCCCATTTTGGGCC	Initial denaturation - 95˚C for 3 min, 32 cycles with initial denaturation 95˚C for 30 sec, annealing - 62˚C for 30 sec, polymerization - 72˚C for 30 sec.
	MS050	rev	TCATACCTTGTAGTAGATGTTT	
GABRA4	MS051	for	ATGGTTTCTGTCCAGAAGGTAC	Initial denaturation - 95˚C for 3 min, 29 cycles with initial denaturation 95˚C
	MS052	rev	TTACATTAGACTTTCTGATTTC	

				for 30 sec, annealing - 61˚C for 30 sec, polymerization - 72˚C for 30 sec.
PRLR	MS053	for	ATGCCATCTGCACTTGCTTTCG	Initial denaturation - 95˚C for 3 min, 27 cycles with initial denaturation 95˚C for 30 sec, annealing - 60.8˚C for 30 sec, polymerization - 72˚C for 30 sec.
	MS054	rev	TCAGTAGTCAAGTTCCCCTGCA	
GAPDH	MS055	for	CAAGGTCATCCATGACAACTTTG	Initial denaturation - 95˚C for 3 min, 22 cycles with initial denaturation 95˚C for 30 sec, annealing - 55˚C for 30 sec, polymerization - 72˚C for 30 sec.
	MS056	rev	GTCCACCACCCTGTTGCTGTAG	
pGL3	AG264	rev	GGTTACAAATAAAGCAATAGCATCACA	
A2bp1	MS001	A2bp1 for	AAAGGCCGGCCATGATAAAACCATTAA ACAAACAAACAAAAAAC	L=478bp, from *Rattus norvegicus* gDNA or cDNA. For cloning into pGL3 with FseI.
A2bp1	MS002	A2bp1 rev	AAAGGCCGGCCGGCCTCCCCTTTCACA GAATAAAATAATATAG	
Creb5	MS003	Creb5 for	AAATCTAGAAAGGCATCGGTCAAACCT GGCC	L=1745bp, from *Mus musculus* gDNA or cDNA. For cloning into pGL3 with XbaI and FseI.
Creb5	MS004	Creb5 rev	AAAGGCCGGCCCTCTAAAATAGAATTT TTTTTAAACTATAGTGAGCG	
MAP3K2	MS011	MAP3 K2 for	AAATCTAGACAGCCAGCGTCCTCCACCC GCC	L=156bp, from *Rattus norvegicus* gDNA or cDNA. For cloning into pGL3 with XbaI and FseI.
MAP3K2	MS012	MAP3 K2 rev	AAAGGCCGGCCTTTAGTTTACTATTATT ATTAAACAAATTTAACCAAG	

3.1.13. miRNA target prediction

miRNA targets for further analysis were predicted using basic seed-based algorithms (Lewis et al., 2005) from the Targetscan database (Whitehead Institute for Biomedical Research, Cambridge, MA, USA; www.targetscan.org). Predicted targets of a miRNA family were calculated as published earlier (Grimson et al., 2007) and sorted by total context score. The total context score was based on the following features: site-type contribution, 3' pairing contribution, local AU contribution, and position contribution (Grimson et al., 2007).

3.1.14. Preparation of E. coli JM109 electrocompetent cells

E. coli JM109 cells (Agilent Technology Inc./Stratagene, Santa Clara, CA, USA) were picked up from fresh LB/agar plates, inoculated into 20 ml of LB and grown overnight at 37˚C with shaking (200 rpm). The next day, 6 ml of overnight culture were inoculated into 300 ml of LB medium and grown at 37˚C with shaking (200 rpm) until OD_{600} = 0.6. Bacterial cells were chilled down on ice for 30 min and collected by centrifugation at +4˚C for 20 min, 4000 rpm. The bacterial pellet was washed 2 times in 50 ml ice-cold sterile de-ionized water, one time in 10% ice-cold glycerol, and resuspended in 3 ml of 10% ice-cold glycerol. Cells were split into 80 µl aliquots and stored at -80˚C.

3.1.15. Cloning

The 3’- untranslated regions (UTR) of the *Eps15* gene (NM_001009424) with a seed sequence for the miR-186 and *Nab1* (NM_022856) genes with a seed sequence for miR-

709 were amplified by PCR. They were then cloned into pGL3-Promoter vectors downstream of the Luciferase coding sequence. The PCR mix contained 10 µl of 5x GC buffer, 5 µl of 2 mM dNTPs, 1 µl of 10 µM forward and reverse primers (see Table 3.1), 1 µl of cDNA, and 0.5 µl of Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA) in a total volume of 50 µl. The PCR conditions were: initial denaturation – 30 sec at 98˚C, 25 cycles with denaturation - 10 sec at 98˚C, annealing – 30 sec at 63˚C, polymerization – 30 sec at 72˚C, and final extension 10 min at 72˚C.

The PCR fragments were analyzed by gel electrophoresis in 1% agarose/1x TAE buffer with ethidium bromide, and purified using a PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR fragments and pGL3-Promoter plasmid were digested with XbaI and FseI restriction endonucleases (NEB, Ipswich, MA, USA). After digestion they were purified using PCR purification kit (Qiagen, Valencia, CA, USA) and ligated overnight at 16˚C. The ligation reaction consisted of 2 µl of 10x ligation buffer, 300 ng of vector DNA, 400 ng of PCR fragments, and 2 µl of T4 DNA ligase (Fermentas, Burlington, ON, Canada) in a total volume of 20 µl. Ligation mixes were purified with phenol:chloroform, precipitated with ethanol, dissolved in ultra-pure water (NEB, Ipswich, MA, USA) and used for transformation of JM109 electrocompetent cells by electroporation (1600 V). After electroporation the transformed JM109 cells were plated on LB/agar plates supplemented with 50 µg/ml of kanamycin. Plates were incubated overnight at 37˚C. Resistant clones were screened by colony PCR using the DreamTaq DNA Polymerase (Fermentas, Burlington, ON, Canada) under the following conditions: initial denaturation - 95˚C for 5 min, 25 cycles with denaturation for 30 sec at 95˚C,

annealing - 55˚C for 30 sec, polymerization - 72˚C for 30 sec, and final elongation at 72˚C for 1 min. PCR fragments were checked by gel electrophoresis in 1% agarose gel/1x TAE with ethidium bromide. Positive clones were transferred onto fresh LB/agar/kanamycin plates and stored at +4˚C. Resulted plasmids (pFN4 that is pGL3::Eps15-3'UTR and pFN7 - pGL3::Nab1-3'UTR) were used for generation of mismatch controls (without proper seed sequence region for miRNAs).

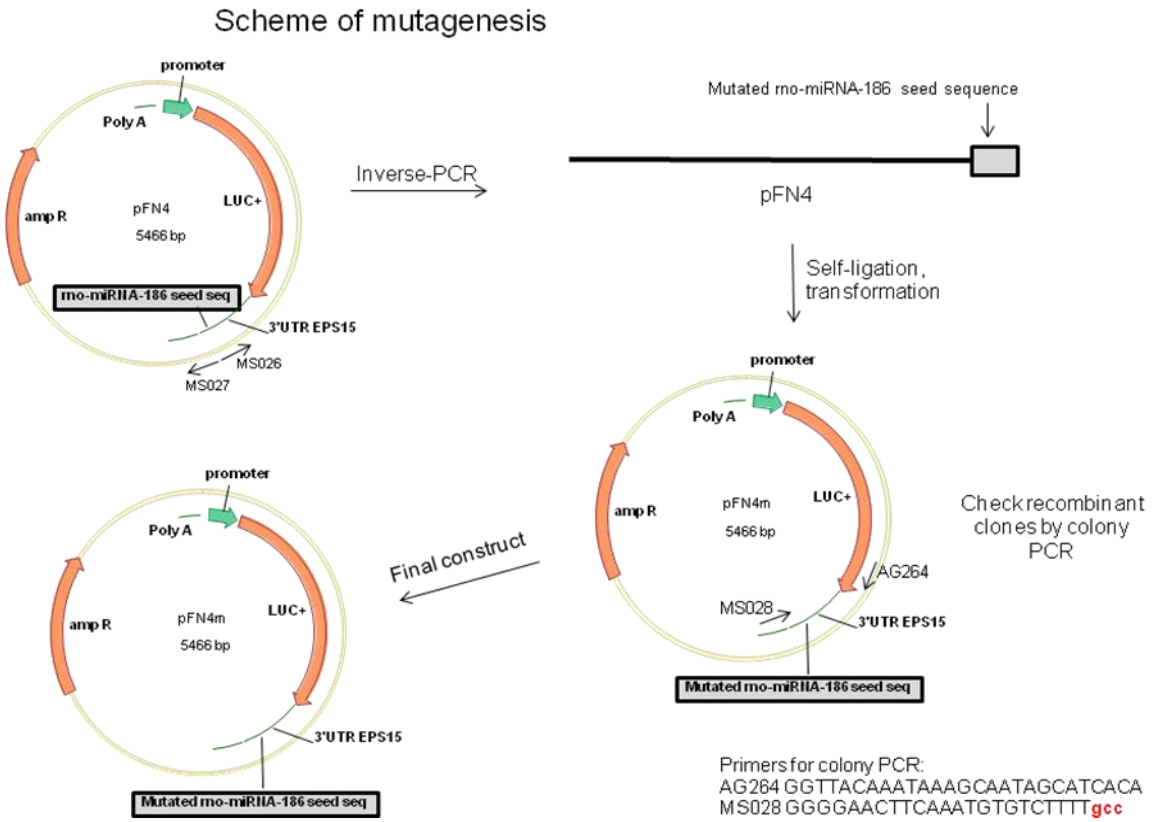
3.1.16. Seed sequence mutagenesis

To create mismatch controls the pFN4 plasmid was mutated with primers MS026 and MS027, and pFN7 with primers MS044 and MS055 that carry mutated seed sequences (see Table 2). PCR fragments were generated with Phusion® High-Fidelity DNA Polymerase (see ***Cloning***), extracted from the 1% agarose gel with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and self-ligated using T4 DNA ligase (Fermentas, Burlington, ON, Canada). Ligation reaction consisted of 2 µl of 10x ligation buffer, 10 µl of gel-purified PCR fragment DNA (10 ng/µl), and 2 µl of T4 DNA ligase in a total volume of 20 µl. The ligation mix was incubated overnight at 16˚C, phenol:chlorophorm was purified and used for transformation of electrocompetent *E. coli* JM109 cells. Mutated plasmids (pFN4mut and pFN7mut) were screened by colony PCR with corresponding primers: pFN4mut – MS028 and AG264 (see Fig.3.4, and Table 3.1), pFN47mut – MS046 and AG264 (Table 3.2). Original and mutated plasmids were confirmed by DNA sequencing.

3.1.17. Cell culture and Luciferase Reporter Assay

Human embryonic kidney HEK-293 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, penicillin (100U/ml) at 37˚C in a 5% CO_2 atmosphere. HEK293 cells were cotransfected in 24-well plates with the pGL3 vector (with Firefly luciferase) or tested construct, precursor miRNA, and control Renilla luciferase pRL-TK vector (Promega, Madison, WI, USA), using the Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Twenty-four hours after transfection, 1x Passive Lysis Buffer (Promega, Madison, WI, USA) was added to the transfected cells. Renilla and Firefly luciferase activities were measured using the dual-luciferase reporter assay system with Stop & Glow Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. A similar culture experiment was performed using another cell line, human breast cancer MCF-7 cells. Each cell line was tested in triplicate and reproduced twice in independent experiments.

A



B

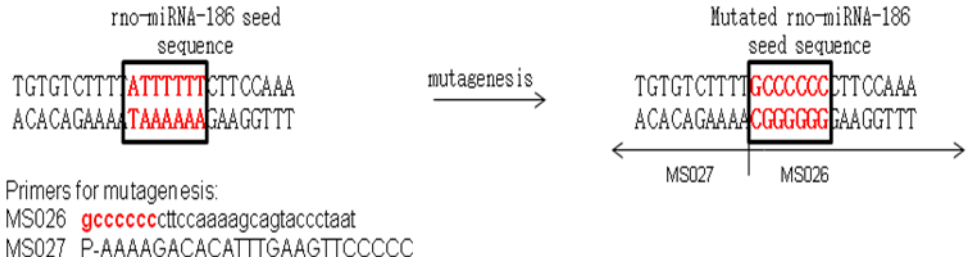


Figure 3.4. A: Schematic illustration of the seed sequence mutagenesis. The PCR fragment with a mutated seed sequence was obtained by inverse PCR with corresponding primers from the original pFN4 plasmid. The PCR fragment was self-ligated and used for transformation of JM109 electrocompetent cells. Recombinant clones were checked by colony PCR. The mutated seed sequence of the final construct was verified by sequencing. B: rno-miRNA-186 seed sequence (highlighted in red) was substituted with the mutated sequence (adenine was substituted by guanine, while thymine was substituted by guanine).

3.1.18. Analysis of genome-wide cytosine methylation

Genomic DNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Cytosine-extension assay to detect sequence-specific alterations in DNA methylation was performed using a protocol by Pogribny et al. (1999). Briefly, 2 µg of genomic DNA was digested overnight with a 10-fold excess of HpaII or MspI endonuclease according to manufacturer's protocol (New England Biolabs, Beverly, MA, USA). A third DNA aliquot was incubated without restriction enzyme used as a background control. The single nucleotide extension reaction was carried out in a 25 µl reaction mixture containing 0.5 µg of DNA, 1x PCR buffer II, 1.0 mM $MgCl_2$, 0.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA, USA), [^{3}H]dCTP (57.4 Ci/mmol) (NEN, Boston, MA) and incubated at 56°C for 1 h, then placed on ice. Duplicate 10 µl aliquots from each reaction were applied on Whatman DE-81 ion-exchange filters and washed three times with Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. Background radiolabel incorporation was subtracted from enzyme-treated samples and the results were expressed as relative [^{3}H]-dCTP incorporation/0.5µg DNA or as percent change from control samples (Pogribny et al., 1999). The data obtained from 3 independent experimental groups with 2 measurements per group were expressed as a percentage of dpm/mg of DNA relative to background controls. Incorporation is directly dependent on the completeness of the DNA digestion with methylation-sensitive enzymes. Higher methylation is associated with a decrease in digestion rate and, thus, a lower incorporation of radioactively labeled cytosine ([^{3}H]-dCTP).

3.1.19. Data analysis and statistics

3.1.19.1. General statistics

Statistical analysis was performed using Microsoft Excel Analysis ToolPak (Microsoft Corp., Redmond, WA, USA). All data are presented as the mean +/- standard deviation. Details of each type of analysis are provided in the following.

3.1.19.2 miRNA microarray analysis

The miRNA microarray data analysis was performed by LC Sciences (Houston, TX, USA). Data analysis included the determination of detectable signals, calculation of signal intensities, and, calculation of differential ratios. The data analysis process began with background subtraction, Cy3/Cy5 channel normalization, detectivity determination, and then p-value calculation for the determination of differential significance. Multiple sample analysis involved normalization, data adjustment, t-test/ANOVA analysis, and clustering.

Background Subtraction. The background was determined using a regression-based background mapping method. The regression was performed on the data points of 5% to 25% of the lowest intensity, excluding blank spots. The raw data matrix was then subtracted from the background matrix.

Normalization. Normalization was carried out using a LOWESS (Locally-weighted Regression) method on the background-subtracted data (Bolstad et al., 2003). The normalization allows removing system-related variations, such as sample amount

variations, different labeling dyes, and signal gain differences of scanners so that biological variations can be reliably revealed.

Detectivity Determination. Detectable transcripts met at least two conditions: signal intensity higher than 3 × (background standard deviation) and spot CV < 0.5. CV was calculated by (standard deviation)/(signal intensity). When repeating probes were present on an array, a transcript was listed as detectable only if the signals from at least 50% of the repeating probes were above detection level.

P-Value Calculation. After the normalization, the p-values of the difference between Cy3 and Cy5 signals were calculated as following. Let *StdCy*3 and *StdCy*5 be the standard deviations Cy3 and Cy5 probe areas, respectively. Let *Cy*3 and *Cy*5 be the signal intensities (the values obtained after background subtraction and normalization) of the Cy3 and Cy5 probe areas. And let *StdBkgCy*3 and *StdBkgCy*5 be the standard deviations of background values of Cy3 and Cy5 channels. Let

$$C = \sqrt{StdBkgCy3^2 + StdBkgCy5^2}$$
$$dferr = \sqrt{StdCy3^2 + StdCy5^2} + C$$

where *C* is a numeral number and *dferr* is a 1D array of size *n*.

Let

$$Stemp = \frac{Cy5 - Cy3}{dferr}$$
$$Stemp = \frac{Stemp - mean(Stemp)}{\sqrt{2}}$$

and

$$p(i) = \frac{2}{\sqrt{\pi}} \int_{Stemp(i)}^{\infty} e^{-t^2} dt$$

If $p(i) < 0.01$, it was plotted as red spot in a log scatter plot.

Probe Standard Deviation. Probe standard deviation was obtained by multiplying Density (st.dev.) by the corresponding normalization correction factor as shown in the following formula:

$$StDev = \sigma \times f$$

P-value. In a dual sample assay, p-value was a measure of statistical significance of signal difference between two samples (Sample A and Sample B) on a detection probe.

Log_2 Ratio. In a dual sample assay, a $\log_2$ ratio was a measure of signal difference between two samples on a detection probe. The $\log_2$ ratio was calculated according to the following formula:

$$Log_2 R = \log_2\left(\frac{I_B}{I_A}\right)$$

where, I_A and I_B are signal count (or intensity) of samples A and B, respectively. One can calculate the corresponding arithmetic ratio either by directly taking $R = I_B / I_A$ or by indirectly converting $\log_2$ ratio using $R = 2^{Log_2 R}$.

Data Adjustment. Data adjustment included data filtering, $\log_2$ transformation, and miRNAs centering and normalization. The data filtering removed miRNAs with (normalized) intensity values below a threshold value of 32 across all samples. The $\log_2$

transformation converted intensity values into the $\log_2$ scale. Gene centering and normalization transform the $\log_2$ values using the mean and the standard deviation of individual genes across all samples using the following formula:

Value = [(Value) – Mean (Gene)] / [Standard deviation (Gene)].

T-test. A t-test was performed between "control" and "stress" sample groups with each group containing at least two samples (Pan, 2002). T-values were calculated for each miRNA, and p-values were computed from the theoretical t-distribution. miRNAs with p-values below a critical p-value ($p<0.01$) were selected for cluster analysis. The clustering was performed using a hierarchical method and was performed with average linkage and Euclidean distance metric (Eisen et al., 1998).

ANOVA (Analysis of Variance). The miRNAs with significant differences in means were picked out across three or more groups of samples, with each group containing at least two samples. In this analysis, p-values were computed from the F-distribution.

All data processes, except the clustering plot, were carried out using in-house developed computer programs. The clustering plot was generated using the TIGR MeV software (Multiple Experimental Viewer, location) from The Institute for Genomic Research (Information provided by Jason Mulcahey, Accounts Manager LC Sciences: www.lcsciences.com).

3.1.19.3. mRNA microarray analysis

mRNA microarray analysis was performed using FlexArray 1.4.1 software (Blazejczyk et al., 2007). The data analysis was performed using the lumi Bioconductor package (Du et al., 2007), which was used for the pre-processing and normalizing of Illumina microarray data. Background correction was performed using Robust Multichip Average (RMA) background adjustment (Bolstad et al., 2003, Irizarry et al., 2003a, Irizarry et al., 2003b). Log_2 data were normalized using Quantile normalization. A two-sample student's t-test was run to compare gene expression in different groups. Data were plotted using volcano plots of p-values. The list of genes 2-fold up- or down-regulated with a p-value of ≤ 0.05 was generated.

3.2. Results

3.2.1. Body weight

Body weight growth curves were not different between the control and the stressed groups (Fig. 3.5 and 3.6).

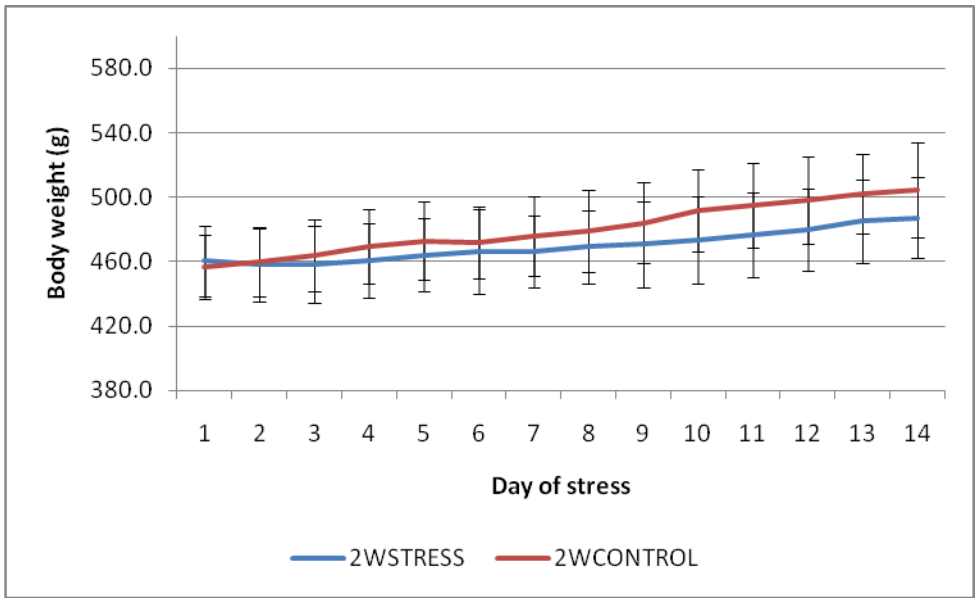


Figure 3.5. Body weight growth curves. The average body weight gain dynamics of stressed animals after two weeks of stress is shown in blue. The average body weight gain dynamics of control animals after two weeks of the experiment is shown in red.

The mean weight of animals in the stressed group (487.3±25.2 g) was 3.5% lower than that of the control group (504.5±29.2 g) (Fig. 3.6).

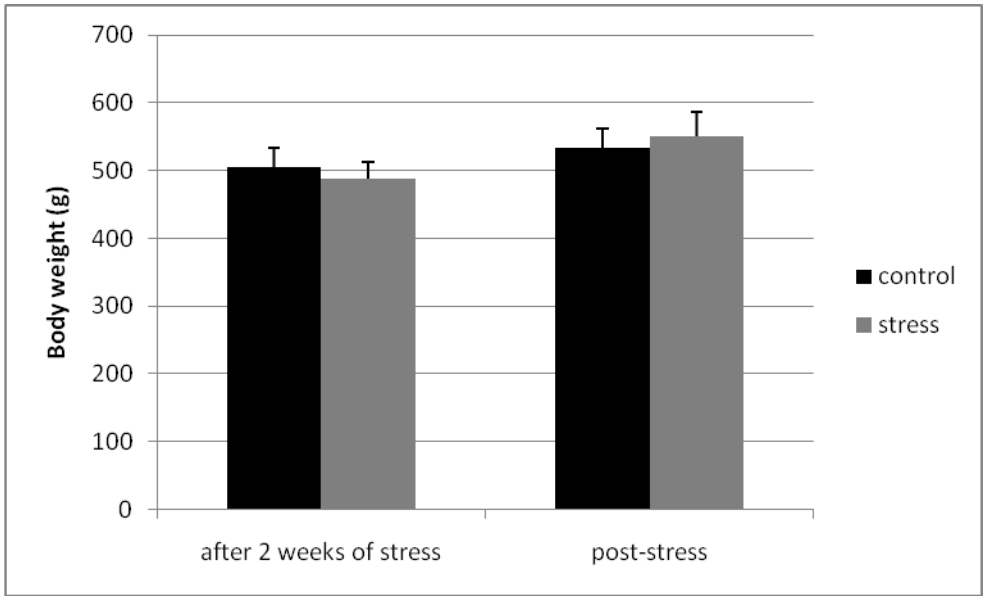


Figure 3.6. Body weight (means ± SD) after two weeks of stress ("after two weeks of stress") and after two weeks of stress followed by two weeks of recovery ("post-stress").

3.2.2. Corticosterone and glucose levels

Analysis of the concentration of plasma corticosterone in control animals and stressed animals showed significant differences ($p<0.001$) on the first day of stress (Fig. 3.7). As expected, on the last day of stress and after recovery from stress, stressed animals had lower levels of corticosterone, which were similar to those in control animals. In contrast, there was no difference in glucose levels on the first day of stress between stressed and control animals (Fig. 3.8). The level of glucose was in general lower on the last day of stress as compared to the first day of stress in both control and stress groups.

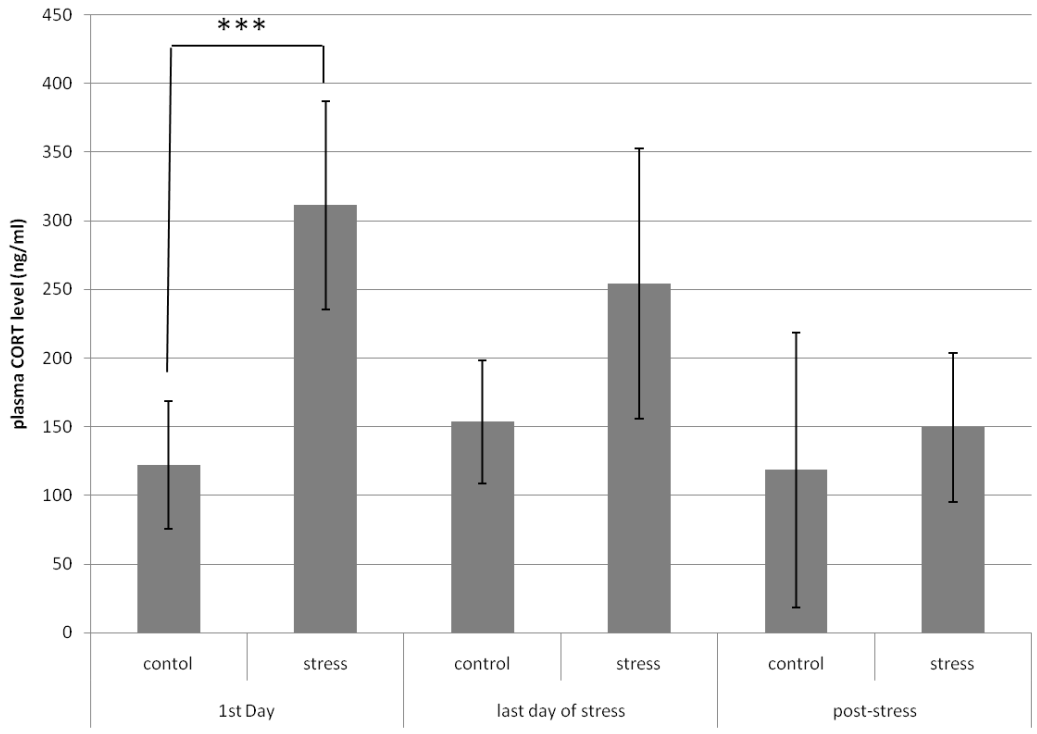


Figure 3.7. Concentration of corticosterone (means ± SD; ng/ml) in plasma in control and stressed animals as measured on the first day of stress, last day of stress and after two weeks of recovery after stress ("post-stress"). Asterisks represent statistical significance ($p<0.001$).

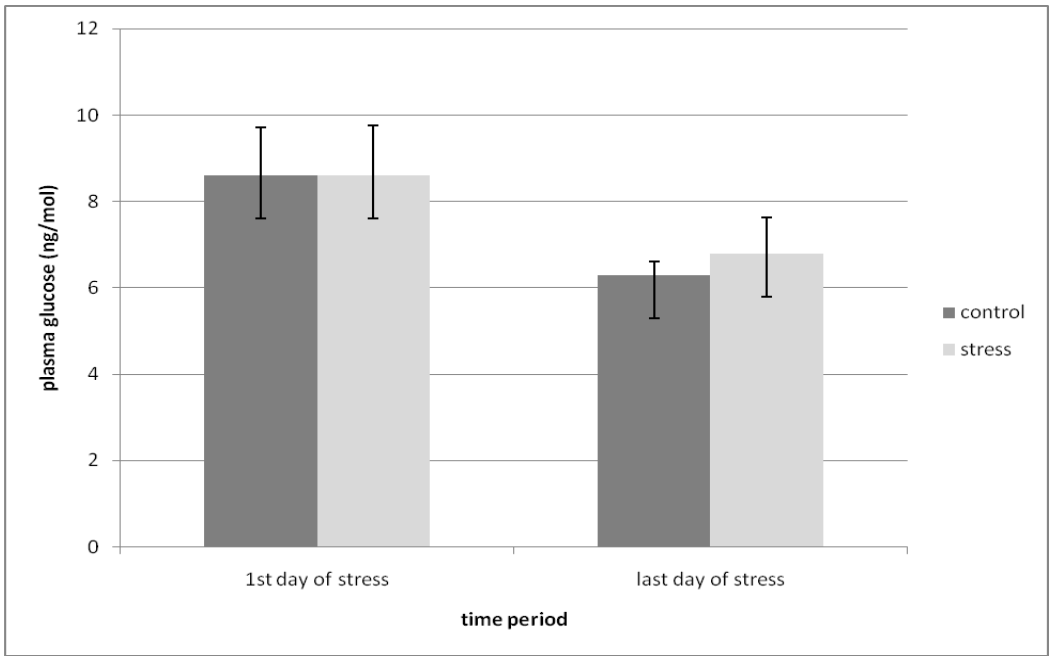


Figure 3.8. Plasma glucose level (means ± SD; ng/ml) in control and stressed animals on the first and last days of stress.

3.2.3. mRNA microarray analysis

We analyzed the mRNA expression pattern in the following experimental groups: 2 weeks of daily restraint stress (2WSTRESS, n=3), 2 weeks controls (2WCONTROL, n=3), 2 weeks of daily restraint stress + 2 weeks of recovery from stress (4WSTRESS, n=3), 4 weeks controls (4WCONTROL, n=3). Three RNA samples out of four per group were chosen for microarray analysis based on RNA quality and concentration. The RatRef-12 Expression BeadChip was used for genome-wide expression analysis that contains 21,910 probes selected primarily from the NCBI RefSeq database (Release 16).

Microarray data analysis showed that after two weeks of restraint treatment (2WSTRESS) 39 genes changed significantly compared to respective controls (2WCONTROL): 36 were up-regulated, while three genes were down-regulated (see Table 3.2, Fig. 3.9). To exclude the possibility that these changes were due to changes in the control animals, we plotted 2WCONTROL vs. 4WCONTROL data, and found only three genes changed, all being up-regulated. The changes observed upon comparison of RNA profile in the 2WSTRESS group of animals vs. 2WCONTROL of animals were nearly completely eliminated after two weeks of recovery after stress; comparison of the expression in the 4WSTRESS group with the expression in the 4WCONTOL group showed just 4 genes were altered and 2 of them were down-regulated. To verify the expression level of genes altered by stress after recovery from stress, we plotted 4WSTRESS vs. 4WCONTOL with fold change < 1.3 and p-value < 0.1 and generated a list of the total number of 803 genes (data not shown). The comparison of expression of 7 overlapping genes is shown in Table 3.3. The comparison of gene expression between the

4WSTRESS group and the 2WSTRESS group showed 12 genes were different, with 4 of them being down-regulated. None of the 12 genes in this comparison were from the above mentioned group of 39 genes.

Table 3.2. List of genes changed after two weeks of restraint treatment (2WSTRESS vs. 2WCONTROL).

Gene ID	log2 (Fold change)	T statistic	P-value
TC2N	1.155517	3.91912	0.01726049
STRA6	1.320069	9.442759	0.000701395
CLCA3_PREDICTED	-1.30728	-4.380687	0.01186854
NUDT7_PREDICTED	1.048149	2.844448	0.04665709
LOC497720	2.586736	5.409068	0.005657964
LOC689246	1.040238	2.907243	0.0437989
RGD1563952_PREDICTED	1.062988	5.394357	0.005713646
PI15_PREDICTED	1.395103	13.8289	0.000158493
CITED1	1.338056	5.607811	0.004967005
LOC500853	1.114951	5.316242	0.006020798
GDF15	1.502859	3.510298	0.02466527
CLDN3	1.517201	3.217527	0.03235646
MGC114520	1.73252	3.273238	0.03069542
ADIPOQ	1.044457	7.537308	0.001659459
RGD1562658_PREDICTED	1.161843	3.426785	0.02661396
LOC502201	1.0398	4.805946	0.008610718
ITGB6	1.058206	6.962303	0.002236928
OSMR	1.101128	8.684738	0.000967566
FOLR1	1.91473	3.050852	0.03799655
LOC361399	1.205812	4.283948	0.01280747
LOC501293	1.445065	2.833147	0.0471942
CLDN2_PREDICTED	2.523236	3.999483	0.01613703
LOC497820	-1.115307	-3.295325	0.03006491
PRLR	1.340845	3.885745	0.01775454
LOC501363	1.120551	4.147749	0.01428635
MSX1	1.636187	4.046863	0.01551584
CDH3	1.007819	4.194682	0.01375455
TCF21	1.14078	2.982395	0.04064249
RGD1564074_PREDICTED	3.106724	4.402334	0.01166997
LOC317599	1.810993	6.90011	0.002313331
CAPN12_PREDICTED	-1.002391	-3.37313	0.02796259
CORIN	1.172354	3.127135	0.03528266
LOC501482	1.026876	3.370437	0.02803238
CRB3	1.132068	3.018464	0.0392224
IQCG	1.041597	4.67263	0.009499686
SLC26A7_PREDICTED	1.187831	3.096842	0.03633221
LOC497864	1.275351	4.161759	0.01412505
OTC	1.589388	2.998462	0.04000258

RGD1563000_PREDICTED	1.406052	4.786004	0.008737058

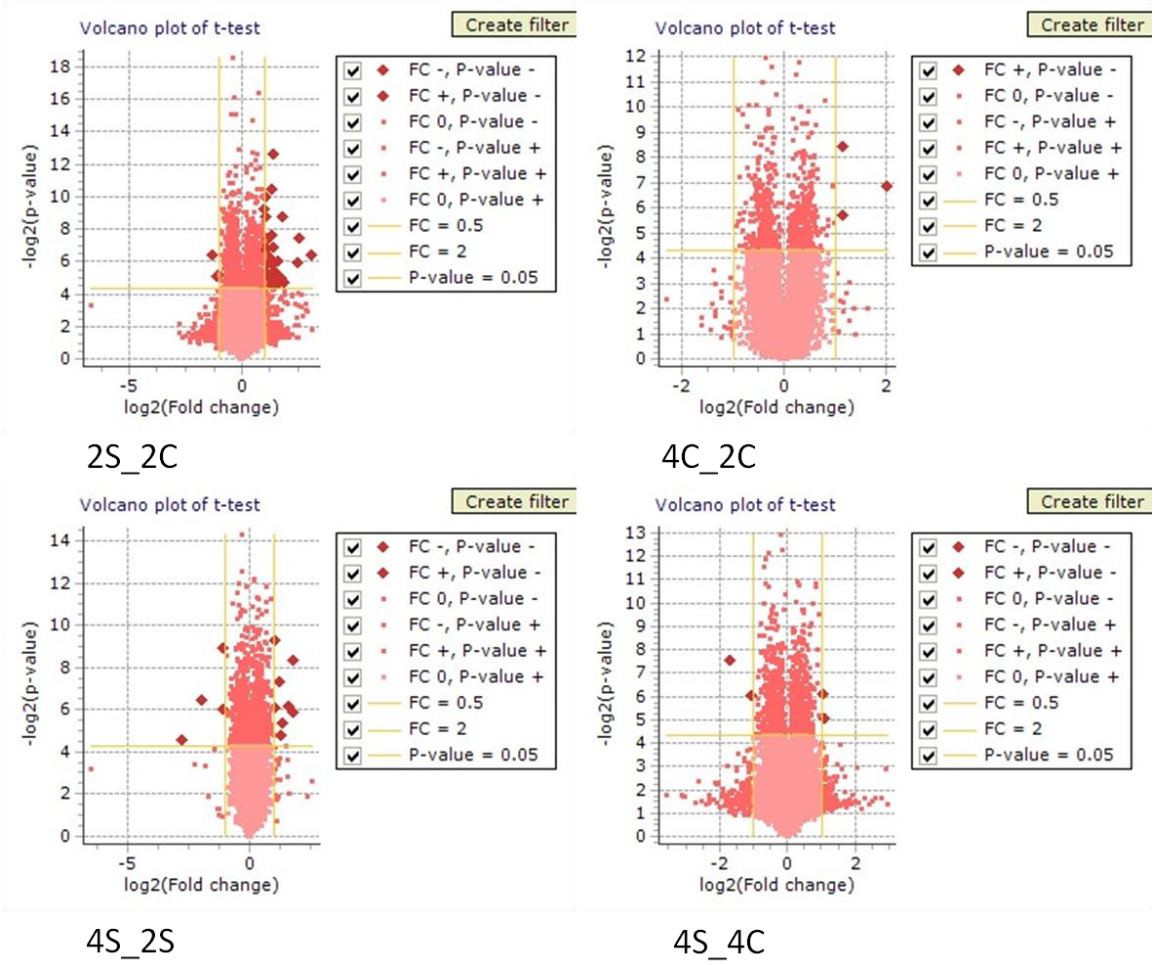


Figure 3.9. mRNA microarray expression analysis. Genes with a 2-fold difference and a p-value of $p<0.05$ are shown. Genes that were changed are represented as dark-red diamonds, in top-left (down-regulated) and top-right (up-regulated) parts of each figure. 2S_2C – groups of 2WSRESS vs. 2WCONTROL, 4C_2C – 4WCONTROL vs. 2WCONROL , 4S_2S – 4WSTRESS vs. 2WSTRESS, 4S_2C – 4WSTRESS vs. 2WCONTROL.

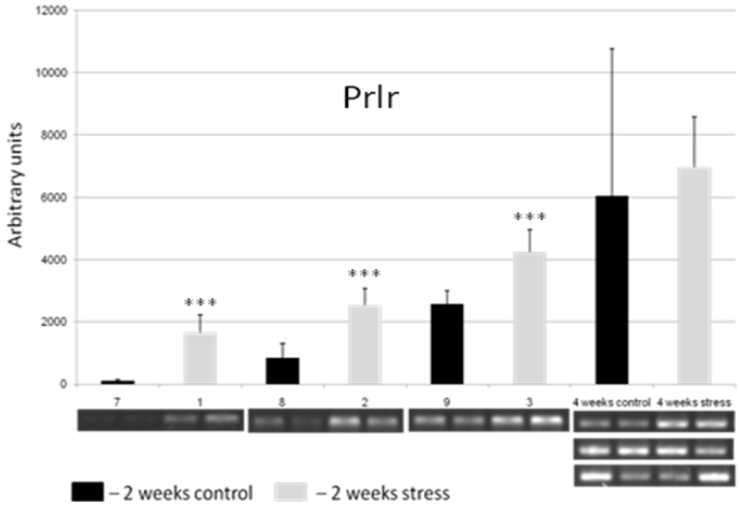
Table 3.3. Comparison of the gene expression levels.

Target ID	2WS vs. 2WC		4WS vs. 4WC	
	log2(Fold change)	**P-value**	**log2(Fold change)**	**P-value**
CRB3	1.132	0.039	0.828	0.088
NUDT7_PREDICTED	1.048	0.047	0.872	0.094
ADIPOQ	1.044	0.002	0.579	0.006
CDH3	1.008	0.014	0.682	0.086
OSMR	1.101	0.001	0.883	0.088
CORIN	1.172	0.035	0.746	0.058
LOC501482	1.027	0.028	0.497	0.045

3.2.4. Semiquantitative RT-PCR

To confirm changes in mRNA expression level we randomly chose two genes from the list of genes that changed after two weeks of stress (Table 3): prolactin receptor (*Prlr*) and adiponectin genes, C1Q and collagen domain containing gene (*Adipoq*). Gamma-aminobutyric acid (GABA) A receptor, alpha 4 gene (*Gabra4*) and ephrin B3 gene (*Efnb3*) were used as controls. sq-RT-PCR was performed in duplicates using the same RNA samples that were used for microarray analysis. Sq-RT-PCR data analysis demonstrated similar levels of RNA expression in comparison to the microarray (more than 2-fold difference in expression level of *Adipoq* and *Prlr*, whereas no significant differences in expression of *Gabra4* and *Efnb3* were observed in the 2WSTRESS vs. 2WCONTROL groups (Fig. 3.10, 3.11). Interestingly, our data showed significant age-dependent changes in the expression level of *Gabra4* and *Efnb3* genes; 4WSTRESS and 4WSTRESS samples showed higher expression than 2WSTRESS and 2WCONTROL (see Fig. 3.11).

A



B

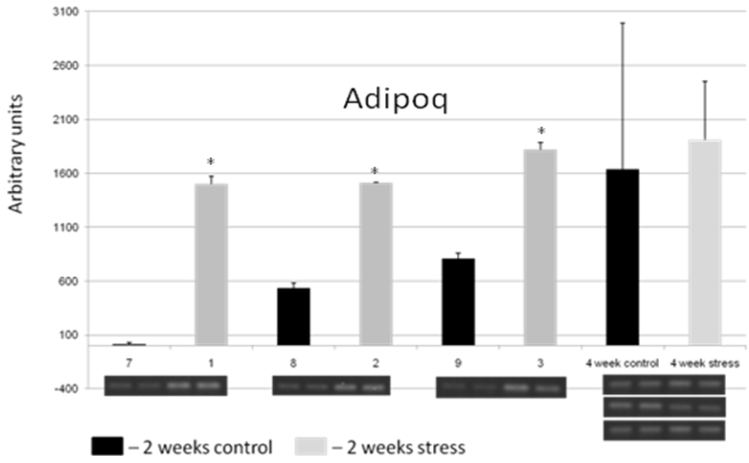
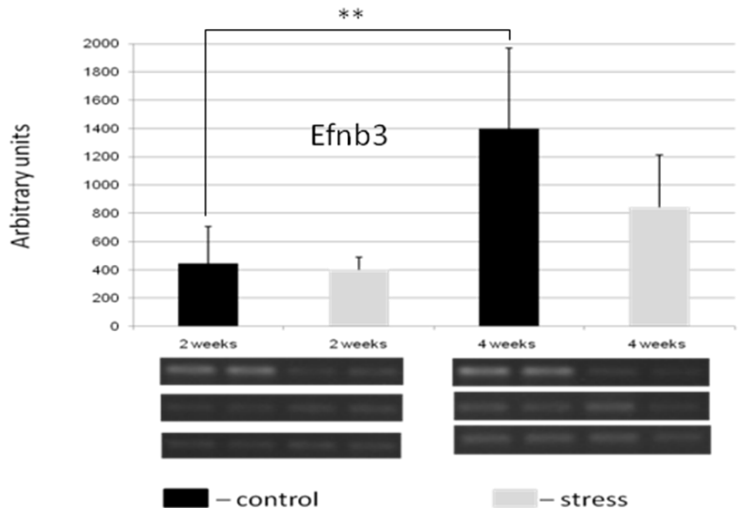


Figure 3.10. sq-RT-PCR analysis. A: prolactin receptor (*Prlr*) gene; B: *Adipoq* gene; Error bars represent standard deviation of the mean. Pictures below bars represent corresponding PCR fragments in duplicates for each animal. Data for 4 weeks are represented as an average for all three animals per group, while 2 weeks data show differences in expression for each animal per group. Asterisks represent statistical significance (*** - $p<0.001$; * - $p<0.05$).

C



D

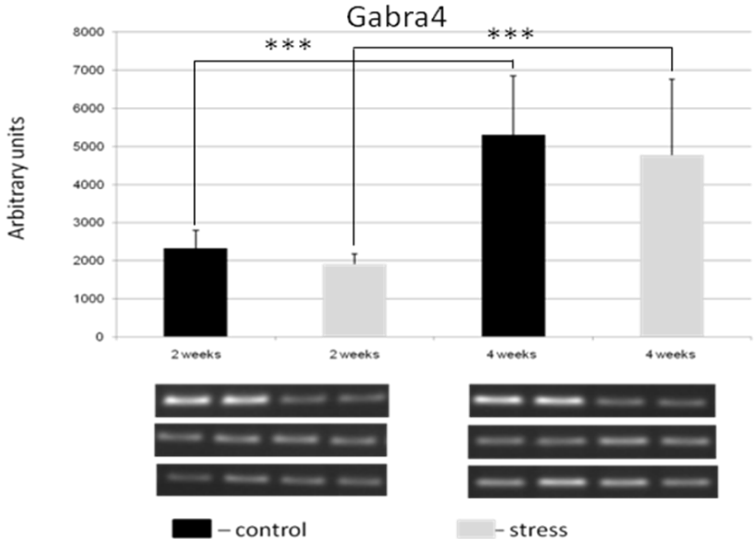


Figure 3.11. sq-RT-PCR analysis. C: ephrin B 3 receptor (*Ef b3*) gene; D: GABA (A) receptor 4 (*Gabra4*) gene. Error bars represent standard deviation of the mean. Data are represented as an average of three animals per group. Pictures below bars represent corresponding PCR fragments in duplicates for each animal for three animals per group. Asterisks represent statistical significance (** - $p< 0.01$; *** - $p<0.001$).

3.2.5. miRNA microarray analysis

miRNA microarray analysis was performed using the µParaflo® Biochip, containing 832 mature miRNA sequences. For analysis the following samples were used: 2 weeks of daily restraint stress (2WSTRESS, n=3) and 2 weeks controls (2WCONTROL, n=3). First, data with p-value p< 0.1 were analyzed. We found that nine miRNAs were changed in stressed animals in comparison to control (see Fig. 3.12). Three miRNAs changed significantly with a p-value p<0.05 in comparison to controls. Two miRNAs, miR-186 and miR-381, were up-regulated, while miR-709 was down-regulated. For further analysis only miRNAs with the lowest p-value (p<0.01) were used, which included miR-186 (fold change 0.43) and miR-709 (fold change -0.66).

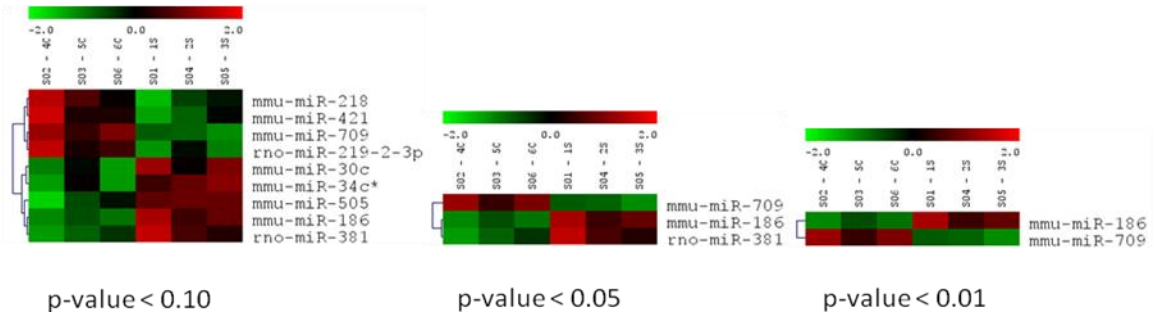


Figure 3.12: Analysis of miRNA expression in the rat cerebellum after two weeks of daily restraint treatment. The microarray heatmap demonstrates the $\log_2$ ratio of miRNA signal difference between control and stress samples. Up-regulated miRNAs are shown in red, while down-regulated miRNAs are shown in green. The first three columns on each figure represent the level of expression in control animals, while the three last columns represent the level of corresponding miRNA expression in stressed animals.

3.2.6. Analysis of predicted miRNA targets

There are no confirmed targets for miR-186 and miR-709 in the brain. Computational analysis of predicted targets for miR-186 revealed 365 putative miRNA targets, with a total of 398 conserved sites and 271 poorly conserved sites, with the score from -1.31 (most favorable) to 0.00. The context score for a specific site is the sum of the

contribution of four features: site-type contribution, 3' pairing contribution, local AU contribution, position contribution. The sum of the context scores for each miRNA was calculated, and the most favorable (i.e., lowest) is displayed. Predicted targets of a miRNA family are sorted by total context score, calculated as in Grimson et al. (2007). We examined the first 50 genes with the most favorable score (<-0.59). Among those we chose five targets which are important in the brain: *Gabra4* (score: -1.31), *Creb3* (-1.07), and *Eps15* (-0.93), *A2bp1 (-0.65), Map3k2 (-0.81).* We were able to clone the wild and mutated seed sequence only of *Eps15* into the plasmid used for Luciferase Reporter Assay, and thus we performed the analysis for *Eps15* only (Fig. 3.13.). The 3'UTR of the *Eps15* gene (NM_001009424) contains one binding site with a poorly conserved seed sequence for rno-miR-186 with one mismatch. The seed sequence is located 422-429 bp downstream of stop codon of *Eps15* CDS. To confirm that *Eps15* is indeed targeted by Mir-186 we carried out a luciferase reporter assay. The 3'UTR of the *Eps15* gene was successfully cloned into the pGL3-promoter vector using the luciferase reporter gene. For the assay we were using MCF-7 and HEK-293 mammalian cell lines and the Dual-Luciferase Assay system (Promega, USA), where the activities of firefly and *Renilla* luciferases were measured from each sample.

Analysis of Luciferase Assay data showed that the luciferase activity was inhibited by miRNA-186 after co-transfection of mammalian cells with the construct carrying *Eps-15* 3'UTR in a dose-dependent manner (Fig. 3.14, 3.16). Co-transfection of mammalian cells with negative control (unrelated miRNA) revealed no changes in

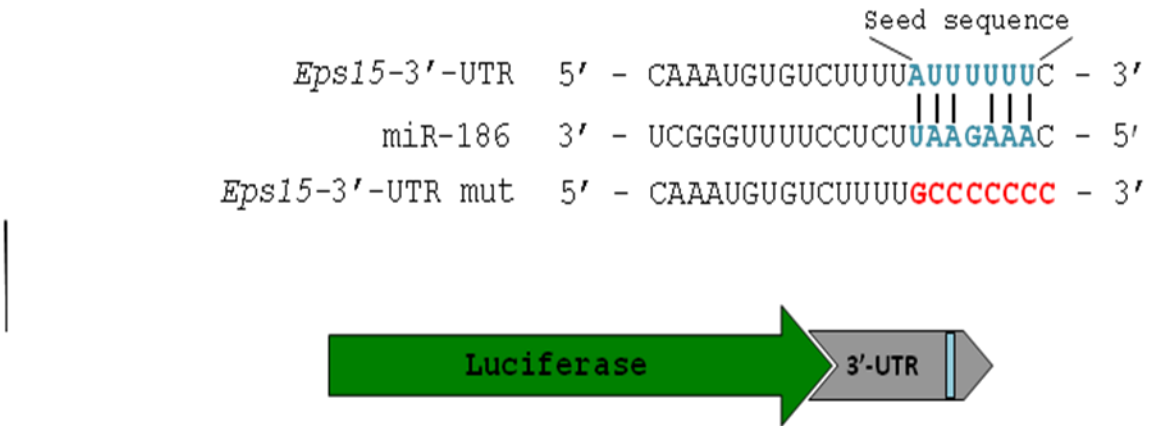


Figure 3.13. Putative binding site of mir-186 in 3'UTR of its target *Eps15*. The seed sequence is represented in blue, while the mutated seed sequence is shown in red.

luciferase activity. The same approach was used to assess the putative target of miRNA-709 (3'UTR of *Nab1* gene). Computational analysis of predicted targets revealed 331 putative miRNA targets, with a total of 347 conserved sites and 193 poorly conserved, with the score from -0.81 (most favorable) to 0.00. We examined the first 50 genes that obtained the most favorable score (<-0.42). Among those we chose four targets which were reported of significant relevance to brain function: *Creb5* (-0.56), *Efnb3* (-0.6), *Nav1* (-0.49), and *Nab1* (-0.43). We were not able to clone wild and mutated seed sequences of *Efnb3, Nav1* and the mutated seed sequence of *Creb5*. We continued further work with *Nab1* only. The 3'UTR of *Nab1* gene (NM_022856) contains one binding site with a poorly conserved seed sequence for rno-miR-709. The seed sequence is located 84-91 bp downstream of stop codon of *Nab1* CDS. Analysis of the Luciferase Assay data revealed no changes in expression of luciferase in the case of normal or mutated seed sequence (Fig. 3.16, 3.17).

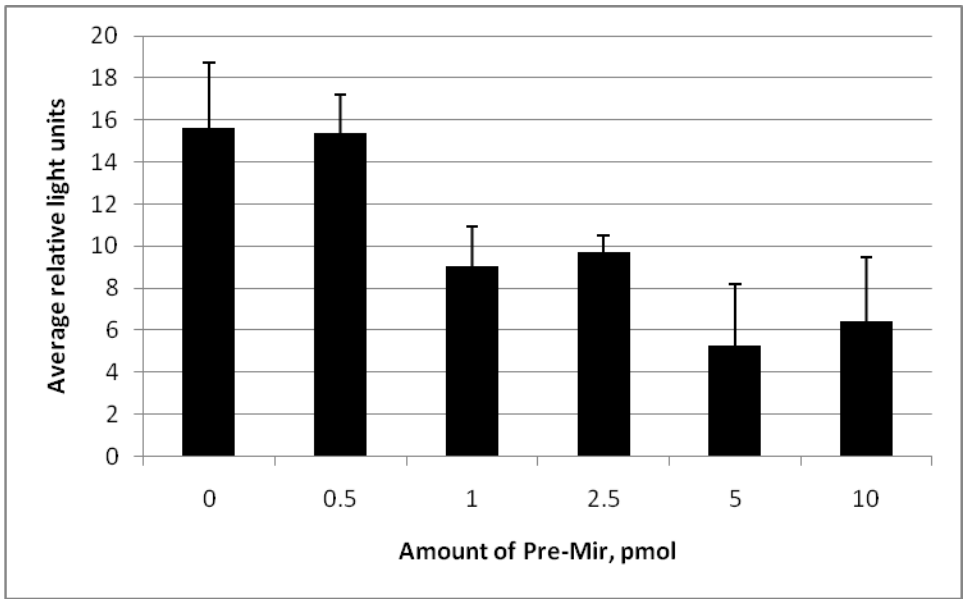


Figure 3.14. Dose-dependent inhibition of *Eps15* expression in the Luciferase Assay after transfection of HEK-293 cells with miR-186. Bars represent the normalized average of relative luciferase units.

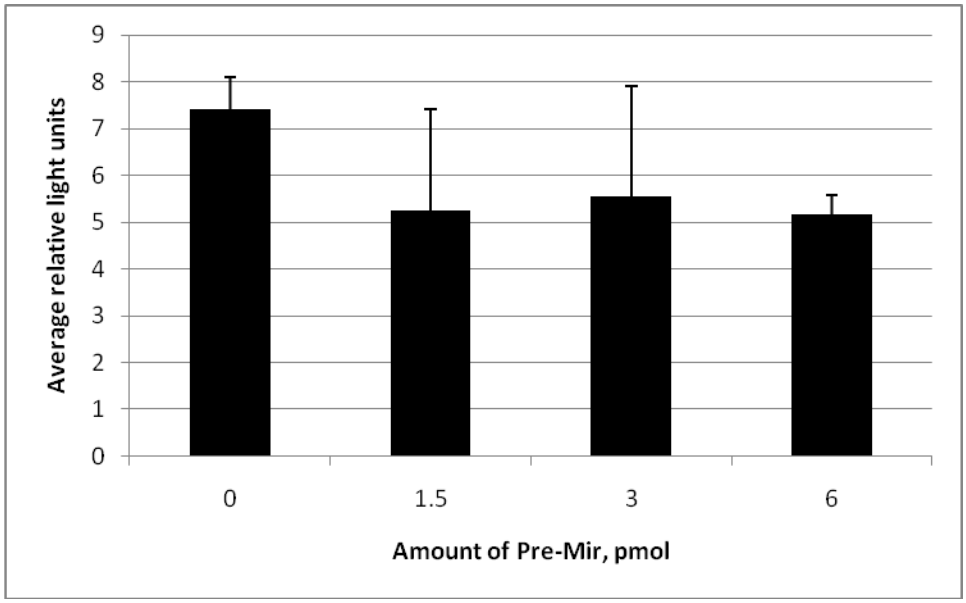


Figure 3.15. Dose-dependent action of *Nab1* gene in the Luciferase Assay after transfection of MCF7 cells with miR-709. Bars represent the normalized average relative of luciferase units.

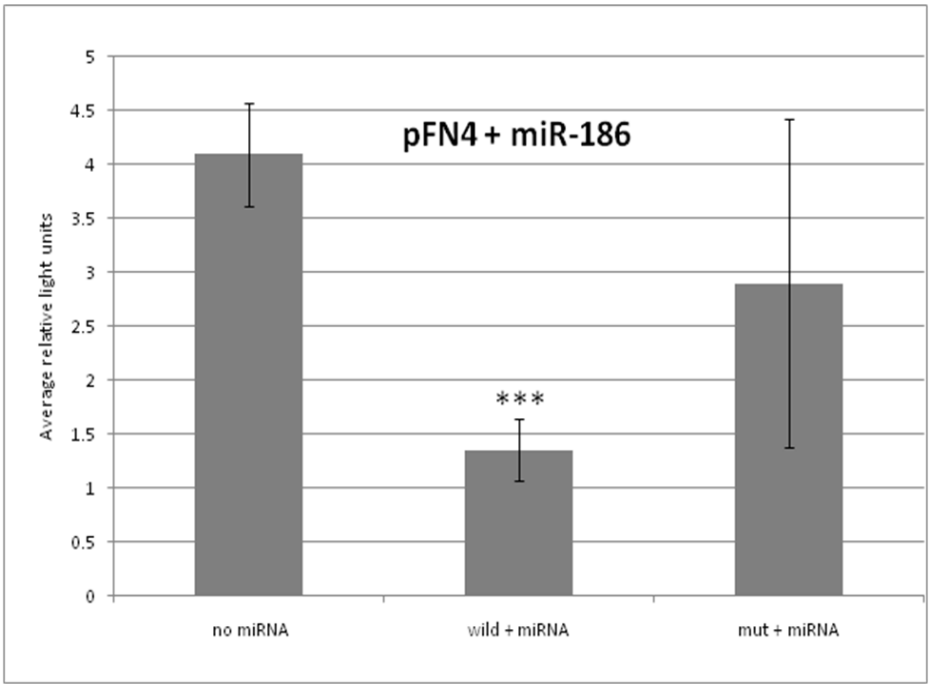


Figure 3.16. Luciferase Assay with pFN4 (3'UTR *Nab1*) and miR-186. The first bar demonstrates relative level of luciferase activity after transfection of MCF-7 cells with pFN4 only. Second bar: pFN4 + miR-186. Third bar: pFN4 mut + miR-186. Bars represent normalized average of relative luciferase units. Asterisks represent statistical significance ($p<0.001$)

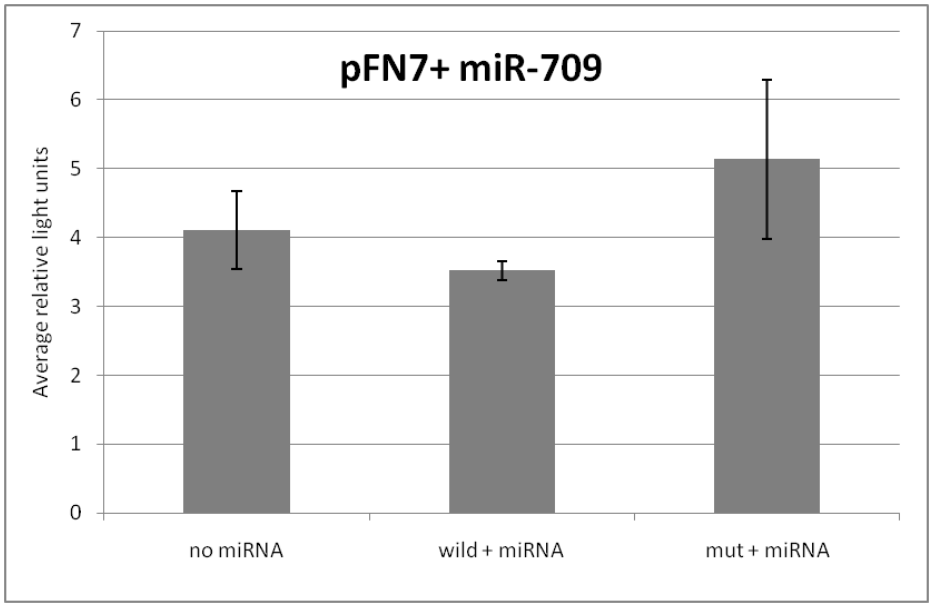


Figure 3.17. Luciferase Assay with pFN7 (3'UTR *Nab1*) and miR-709. The first bar demonstrates relative level of luciferase activity after transfection of MCF-7 cells with pFN7 only. Second bar: pFN7 + miR-709. Third bar: pFN7 mut + miR-709. Bars represent the normalized average of relative luciferase units.

3.2.8. Cloning

In order to confirm putative miRNA targets we needed to clone 3'UTRs of miRNA predicted targets. The choice of 3'UTRs is explained above in **Analysis of predicted miRNA targets**. We have successfully cloned 3'UTRs of the following genes: *Eps15, Nab1, A2bp1, Creb5, Map3k2.* 3'UTR of *Eps15* was cloned under GFP and Luciferase reporter genes. 3'UTRs of *Map3k2, A2bp1,* and *Creb5* were cloned under the GFP reporter gene, while 3'UTR of *Nab1* was cloned under the Luciferase reporter gene (see table 3.4). For colony PCR results see supplemental materials.

Table 3.4. Cloned 3'UTRs

Name	3'UTR	Background plasmid	miRNA
pFN4	*Eps15*	Luciferase reporter	miR-186
pFN7	*Nab1*	Luciferase reporter	miR-709
pGK4	*Eps15*	GFP reporter	miR-186
pGK6	*Map3k2*	GFP reporter	miR-186
pGK1	*A2bp1*	GFP reporter	miR-186
pGK2	*Creb5*	GFP reporter	miR-709

3.2.9. Analysis of genome-wide cytosine methylation

Analysis of the level of genome-wide cytosine methylation was done to asses if observed differences in the gene expression patterns after restraint treatment were caused by differences in the genome-wide methylation. Analysis revealed no changes in the level

of genome-wide cytosine methylation between stress and control groups of animals (Fig. 3.18).

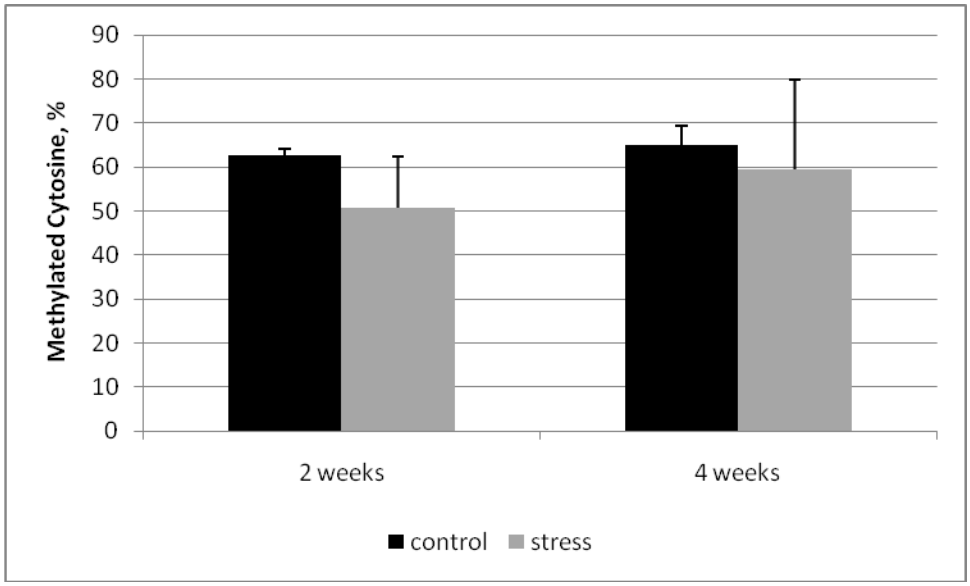


Figure 3.18. Genome-wide cytosine methylation. The "Y" axis shows the percentage (mean ±SD) level of genome-wide cytosine methylation in cerebellum of animals after two weeks ("2 weeks") of stress and two weeks of recovery after two weeks of stress ("4 weeks").

3.3. Discussion

The purpose of this research was to quantify the extent of epigenetic adaptation to a chronic mild stressor in the cerebellum using a rat model. mRNA and miRNA expression profiles were assayed in animals exposed to two weeks of daily restraint stress and two weeks after cessation of stress in comparison to no-stress controls. The results revealed that chronic stress caused changes in gene and miRNA expression in the cerebellum. Furthermore, we verified one putative target for one of the changed miRNAs and expression of four randomly selected genes.

3.3.1. Physiological and behavioural changes

Chronic restraint stress in the present study caused elevated CORT levels on the first day of stress, which is in line with previously reported data (Magarinos and McEwen, 1995b, Faraday, 2002, Kirkland et al., 2008). Furthermore, we observed that animals show habituation to stress by 14 days of daily restraint treatment. The lack of chronic elevation is in accordance with the notion that rats may habituate to stress across subsequent exposures. For example, Magarinos and colleagues reported that daily chronic restraint stress caused a significant habituation by day 21 in the corticosterone response (Magarinos and McEwen, 1995a).

Our experiment also revealed that there were no differences in body weight gain after two weeks of stress in the experimental group. There also were no differences between stress and control animals after two weeks of recovery after stress. These results are consistent with previous results showing an absence of body weight gain after two

weeks of restraint treatment (Jadavji and Metz, 2008). However, sex differences were observed during the post-stress period in that males gained significantly more weight than females. Moreover, Jadavji and Metz (2008) showed corresponding behavioural changes in motor performance. Interestingly, males did not recover from stress-induced impairment during a post-stress recovery period of two weeks. We hypothesized that the long-lasting disturbances in motor performance in male rats might be caused by epigenetically regulated changes in gene expression in the cerebellum. The results in the present study revealed that chronic stress indeed causes changes in the gene expression pattern in the cerebellum. However, the role of the genes which changed after restraint treatment in the motor performance still needs to be investigated. For further studies we can use a few approaches. To begin with, we can treat rats with artificial miRNAs that will target transcripts of changed genes and asses changes in motor performance. As an alternative, we can treat rats with antibodies against target proteins and monitor differences in rat behaviour. As a long-term goal, we can try to generate knocked-out or knocked-down rats/mice with mutated genes important for motor functions and/or behaviour.

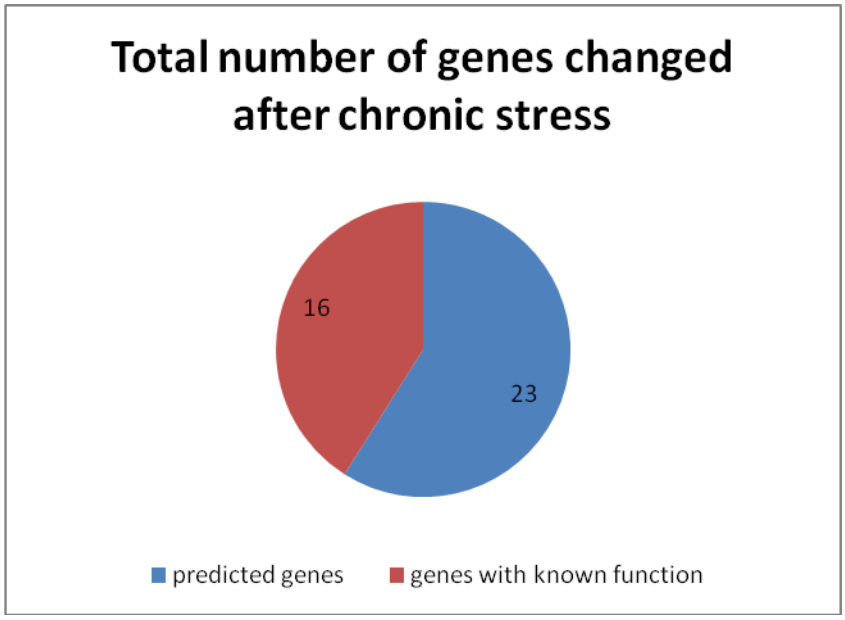
3.3.2. Chronic restraint stress causes changes in mRNA expression in cerebellum

Our results revealed that chronic restraint stress causes changes in gene expression in the cerebellum. After two weeks of restraint treatment we observed up-regulation in 36 genes, while three genes were down-regulated: *Clca3* predicted, LOC497820, *Capn12* predicted. Among the total number of changed genes 23 belong to the category of predicted genes. For further analysis we considered only the remaining 16

genes with known function. For a summary of diseases associated with the altered genes see supplemental materials (Table S8). Interestingly, 14 out of 16 genes were reported to be involved in different types of cancer (Fig. 3.19).

The following will discuss some of the most prominent mRNA and miRNA changes in detail.

A



B

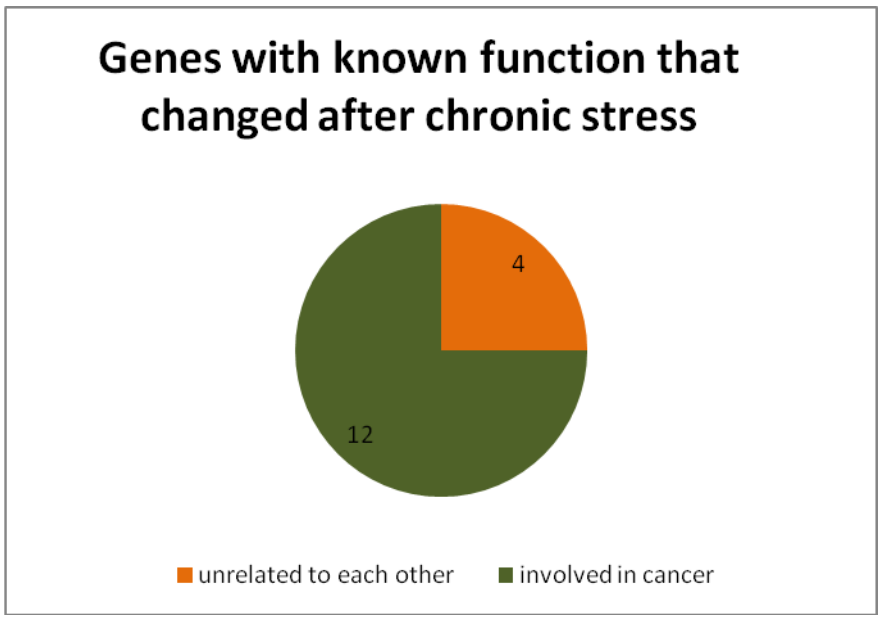


Figure 3.19. Gene classification. A: Total number of genes changed after two weeks of stress. B: Genes with confirmed expression.

3.3.2.1. Adipoq

Our results showed a two-fold difference in expression of *Adipoq*. *Adipoq* is a gene which encodes adiponectin protein, which circulates in the plasma and is important in glucose and lipid metabolism (Qi et al., 2004). It is expressed exclusively in white adipose tissue and is abundantly present in human plasma (Liu et al., 2007). Low levels of adiponectin are associated with diabetes and there is a negative correlation between adiponectin and glucose levels in plasma (Tsao et al., 2002, Qi et al., 2004, Wang et al., 2010). It was reported that adiponectin activates three downstream pathways: AMPK phosphorylation, PPAR-α and p38-Mitogen-Activated Protein Kinase (MAPK) in liver and skeletal muscle, where it has the highest expression (Kadowaki and Yamauchi, 2005, Yoon et al., 2006).

The function of adiponectin in the brain is poorly understood, however, adiponectin receptors have been detected in the brain (Yamauchi et al., 2003). In particular, Yamauchi et al. (2003) demonstrated that the adiponectin receptors *AdipoR1* and *AdipoR2* are expressed in the hypothalamus to mediate glucose uptake by adiponectin (Yamauchi et al., 2003). Adiponectin receptors are expressed ubiquitously throughout the body (Kubota et al., 2007). Adiponectin was reported to mediate increased AMP kinase activity in the hypothalamus and stimulate food consumption (Yamauchi et al., 2003, Kubota et al., 2007). It has been shown that in leptin-deficient obese mice, adiponectin acts in the brain to reduce plasma glucose by 71%, insulin by 52%, triglycerides by 17% and total cholesterol by 29% (Qi et al., 2004, Hoyda, 2009).

Up to date there exists controversy regarding adiponectin expression in the brain. In the rat model experiments, the level of adiponectin was increased in the cerebrospinal fluid (CSF), but not in the plasma after intravenous injection (Qi et al., 2004), suggesting that the brain might be an important target for this hormone. According to Spranger et al. (2006), adiponectin is not expressed in the CNS (Spranger et al., 2006). In their experiments they failed to detect adiponectin in human CSF samples and also did not find evidence of adiponectin crossing the blood brain barrier (BBB) (Spranger et al., 2006). However, the authors showed that brain endothelial cells express adiponectin receptors (Spranger et al., 2006). Nevertheless, several studies reported that adiponectin exists in human CSF at a 0.1% of serum concentration (Kos et al., 2007, Kusminski et al., 2007).

Here we provided the first evidence that Adipoq mRNA is expressed in the nervous system. It is very unlikely that translation-capable Adipoq mRNA will reach the brain with plasma without being degraded. Moreover, it is not known if the molecule is actually able to cross the BBB. Spranger et al. (2006) reported that adiponectin mRNA was not present in brain microvessels harvested from mice (Spranger et al., 2006). Therefore, our data strongly suggest that *Adipoq* mRNA detected in cerebellum should be accounted for by local expression.

Aside from direct effects via central receptors, adiponectin may also act through cell adhesion molecules. Interestingly, it has been proposed that adiponectin binds to the T-Cadherin molecule (Hug et al., 2004). T-Cadherin can actively bind to some forms of adiponectin, suggesting its role as a possible part of a more complex signaling structure

(Hug et al., 2004, Hoyda, 2009). T-Cadherin belongs to a large family of proteins involved in calcium mediated cell–to-cell interactions and extracellular signaling (Hug et al., 2004). The function of T-Cadherin in the adiponectin system remains to be elucidated (Hoyda, 2009). Intriguingly, in our experiments we have observed that increased expression of the *Adipoq* gene occurred simultaneously with the up-regulation of the predicted *Cdh3* gene in the cerebellum. *Cdh3* is a P-Cadherin gene which is overexpressed in the majority of pancreatic cancer, but not in healthy cells (Imai et al., 2008). Imai et al. (2008) proposed that *Cdh3* is a novel tumor-associated antigen, which can be used in cancer immunotherapy (Imai et al., 2008). Taking into consideration that experiments in mice suggest a role of *AdipoR2* in pancreatic islet cell maintenance (Liu et al., 2007, Hoyda, 2009), there might be some connection between *Cdh3* and *Adipoq* functions in the process of developing pancreatic cancer.

Among other functions adiponectin is also implicated in regulation of blood pressure. Tanida et al. (2007) reported that adiponectin decreases blood pressure and sympathetic nerve activity in rats in a dose-dependent manner (Tanida et al., 2007).

Interestingly, it was recently shown that adiponectin might be implicated in the pathophysiology of autism (Fujita-Shimizu et al., 2010). Mori and colleagues showed that serum levels of adiponectin in subjects with autism were significantly lower than those of normal controls (Fujita-Shimizu et al., 2010). Thus, we can conclude that functions of adiponectin are diverse and its specific role in the brain remains to be further investigated.

3.3.2.2. Prolactin receptor (PRLR)

The prolactin receptors are abundant in most tissues, with the highest expression in the liver, mammary glands, adrenal glands and hypothalamus (Brandebourg et al., 2007). Our results demonstrated that chronic restraint stress resulted in a two-fold increase in expression of prolactin receptor mRNA in the cerebellum. These findings are in consistency with previous results reporting that restraint stress in water causes up-regulation of the PRLR in the brain, specifically in the choroid plexus (Fujikawa et al., 1995).

Prolactin (PRL) is a hormone produced by the pituitary, which is closely associated with the stress response (Fujikawa et al., 2004, Brandebourg et al., 2007). Torner et al. (2001) hypothesized that prolactin might play a role in emotional responses and HPA axis reactivity (Torner et al., 2001). Evidence from their experiments in a rat model suggests that prolactin is a neuromodulator of behavioural and neuroendocrine stress-responses, since it has central actions as an endogenous anxiolytic and anti-stress agent (Torner et al., 2001). PRL functions were also implicated in reproduction, development, metabolic and immune processes, brain function and behaviour (Brandebourg et al., 2007). The results by Fujikawa et al. (1995) suggest that in response to restraint stress PRL levels increase and move from the circulation to the CSF, where it acts on the central nervous system (Fujikawa et al., 1995). These authors also demonstrated that circulating PRL causes prolactin receptor expression in the

hypothalamus, suggesting a preventive role against stress-induced hypocalcemia and ulcerogenesis (Fujikawa et al., 1995).

Interestingly, Ben-Jonathan and colleagues (2003) discovered that PRL and PRLR are produced in human adipose tissue (Zinger et al., 2003). Since examination of adipose tissue from rodents and murine pre-adipocyte cell lines failed to detect PRL expression and release, the Ben-Jonathan group concluded that expression of PRL in tissues other than the pituitary is unique to humans and primates (Brandebourg et al., 2007).

3.3.2.3. EphrinB3 and GabaA4 receptors

EphrinB3 Receptors. The ephrin receptors represent a large family of receptor protein tyrosine kinases, which play a crucial role in neuronal survival, axonal pathfinding and establishing neuron-target connections during embryonic development (Willson et al., 2006). The expression of ephrin receptors in the CNS is higher in embryos than in adults and differs depending on the type of receptors (Carpenter et al., 1995, O'Leary and Wilkinson, 1999). For instance, an abundance of EphrinA receptors were shown to be expressed in the adult CNS, while expression of only a few EphrinB receptors was found in the adult brain (Willson et al., 2006). There is a controversy about the expression of mRNA and protein of EphrinB3 receptor in the adult brain, although Ephrinb3 mRNA expression was reported in several adult mouse tissues, including whole brain and adult rat spinal cord (Ciossek et al., 1995, Miranda et al., 1999, Willson et al., 2006). The results of Willson et al. (2006) demonstrated that EphrinB3 receptors (both mRNA and proteins) are abundantly expressed throughout the adult rat brain, with the

most prominent expression in the cerebellum, suggesting that its functional role expands beyond embryonic development (Willson et al., 2006). EphrinB3 receptors and their ligands might play a role in maintaining formed axonal connections and synapses, as well as regulate synaptic plasticity in the mature nervous system (Willson et al., 2006).

Interestingly, our results showed up-regulation of ephrinB3 mRNA expression in two week groups (2WSTRESS + 2WCONTROL) in comparison to our four week groups (4WSTRESS + 4WCONTROL) (Fig. 3.11.C), while there were no changes in expression after stress treatment. We found up-regulation of *Efnb3* mRNA in two weeks control animals as compared to four weeks animals. We speculate that there is an age-dependent effect on its expression. Similarly to EphrinB3, the expression of GABA (A) receptor 4 also changed in two and four week groups of control animals (Fig. 3.11.D).

Gabra4 receptors. γ-Aminobutyric acid (GABA) receptors are signaling proteins that represent the major inhibitory neurotransmitter receptors in the central nervous system (Sieghart et al., 1999). It was reported that the GABA (A) receptor 4 gene (*Gabra4*) could contribute to autism susceptibility in humans (Ma et al., 2005, Collins et al., 2006). Chugani et al. (2001) have observed age-related changes in the distribution of the GABA (A) receptors in the brain of epileptic children (Chugani et al., 2001). Their results demonstrated the highest expression of GABA receptors in the brain at the youngest age measured (2 years), which decreased exponentially with age (Chugani et al., 2001).

Age-dependent differences were also reported for the distribution across the brain of various GABA (A) receptor subunits in rats (Laurie et al., 1992, Poulter et al., 1992, Davis et al., 2000). Studies by Laurie et al. (1992) examined the embryonic and postnatal expression of 13 GABA (A) receptor subunit genes in the rat CNS, showing that each subunit exhibits a unique regional and temporal developmental expression profile (Laurie et al., 1992). For instance, the expression of GABA (A) subunit 4 had its peak at postnatal day 12, whereas subunit 1 and 6 mRNA increased with age, in contrast to subunits 2 and 3 which were down-regulated in cerebellum (Laurie et al., 1992).

3.3.3. Chronic restraint stress causes changes in miRNA expression in the cerebellum

There were also changes in miRNA molecules whose functions are not well known yet. In particular, miR-186 and miR-709, which changed significantly in response to stress, do not belong to those which are abundantly expressed in the brain, and particularly in the cerebellum. There is a lack of previous data on the expression of these miRNAs while their functions and verified targets are still unknown. However, miR-186 was reported to be expressed by postnatal oligodendrocyte lineage cells (Lau et al., 2008). Our experiment revealed that miR-186 can target *Eps15* in mammalian cells. Nowadays there is no agreement on the specific rules of target recognition by miRNA. Some authors suggest that it requires a perfect match between the seed sequence and the miRNA binding site (Rodriguez-Lebron and Paulson, 2006), while others speculate that a nearly perfect match in the seed sequence is enough for target recognition (Ioshikhes et

al., 2007). We have found that for the efficient regulation of a target gene (*Eps15*) the seed sequence miR-186 can have one mismatch.

3.3.4. Epidermal growth factor receptor pathway substrate 15

Epidermal growth factor (EGF) receptor pathway substrate 15 gene (*Eps15*) was first identified as an endogenous substrate for the EGF1 receptor kinase (Fazioli et al., 1993). *Eps15* is believed to have an important role in vesicular traffic, but its exact function is still unknown (Cupers et al., 1997). There is some evidence that *Eps15* may play a role in the clathrin-mediated endocytosis of synaptic vesicle membranes (Chen et al., 1998). It was shown that *Eps15* is concentrated in the presynaptic nerve terminals in rat brain, suggesting a role in the molecular rearrangement of the clathrin coats (Chen et al., 1998). Some data also suggest that *Eps15* might be important for mitogenic signaling (Salcini et al., 1999). There are some human pathologies associated with *Eps15* such as neuroblastoma and acute lymphoblastic and myeloid leukemias, since *Eps15* is located on chromosome 1p31-p32, a region with several non-random chromosomal abnormalities (Salcini et al., 1999).

3.3.5. Analysis of genome-wide cytosine methylation

Analysis of the level of genome-wide cytosine methylation revealed no changes between stress and control groups of animals. Though, the level of methylated cytosines appeared to be in the range of 50-80%, which is consistent with the reported normal methylation level for mammals (Razin and Riggs, 1980). Methylation data are not conclusive because they represent mean numbers for two animals per group. Two other

data points per group were excluded from the analysis because of a broken scintillation counter.

3.4. Conclusions and Future Perspectives

In conclusion, our results show that chronic restraint stress changes mRNA and miRNA expression in the cerebellum. The expression of 39 genes was changed significantly after two weeks of restraint treatment. We confirmed the expression of four randomly selected genes. We also demonstrated that stress changes the miRNA expression pattern in the cerebellum. In particular, *Eps15* was confirmed as a target for one of the significantly changed miRNAs, miR-186.

The present study was focused mainly on the miRNA component of epigenetic regulation of the stress response in the cerebellum. It would be interesting to assess other epigenetic changes, such as DNA methylation and histone modifications in the cerebellum as well as in other brain areas. Our results showed that stress causes alteration in the expression of prolactin receptor in male rats. Since prolactin plays an important role in lactation, it would be interesting to assess sex differences in the stress response.

It would be interesting to investigate the role of the genes which changed after restraint treatment in the motor performance. We can treat rats with artificial miRNAs that will target transcripts of changed genes and asses changes in motor performance. As an alternative, we can treat rats with antibodies against target proteins and monitor differences in rat's behaviour. As a long-term goal, we can try to generate knocked-out or knocked-down rats/mice with mutated genes important for motor functions and/or behaviour.

Supplemental Materials

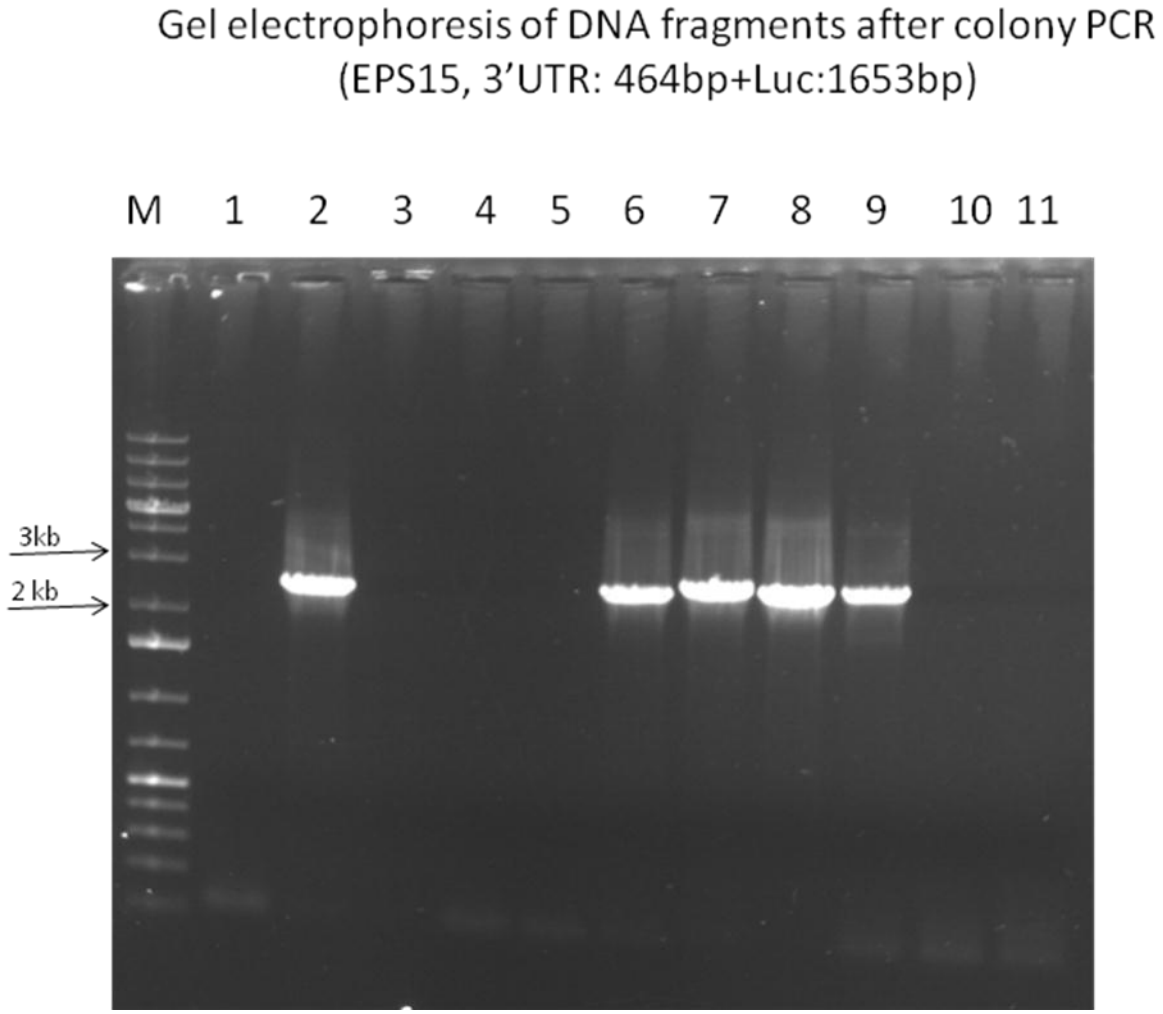


Figure S1. Gel electrophoresis of DNA fragments after colony PCR for pFN4. Each PCR fragment represents one positive recombinant clone.

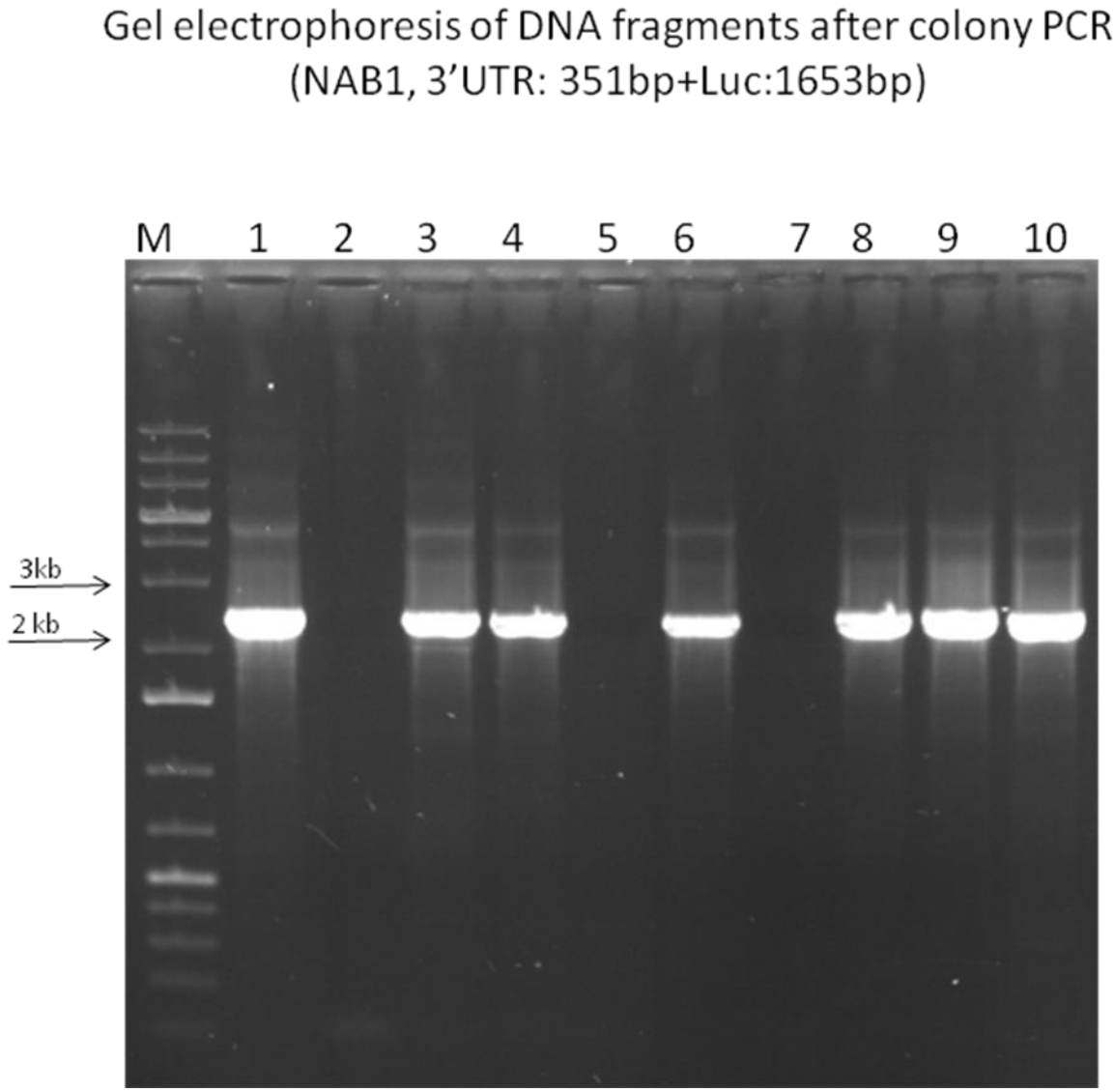


Figure S2. Gel electrophoresis of DNA fragments after colony PCR for pFN7. Each PCR fragment represents one positive recombinant clone.

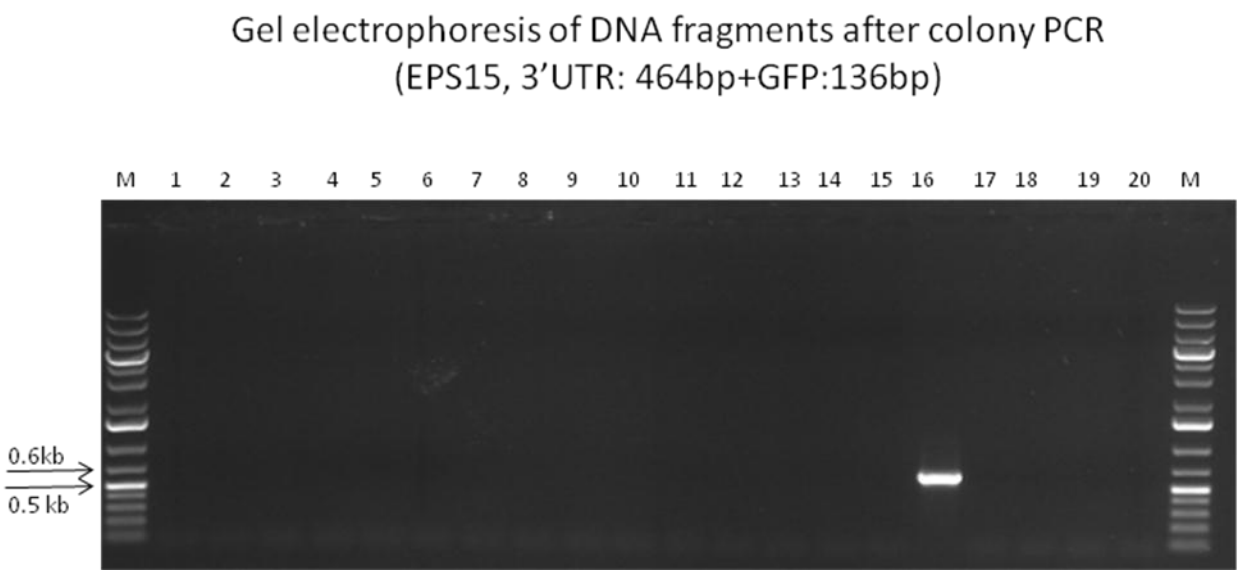


Figure S3. Gel electrophoresis of DNA fragments after colony PCR for pGK4. Each PCR fragment represents one positive recombinant clone

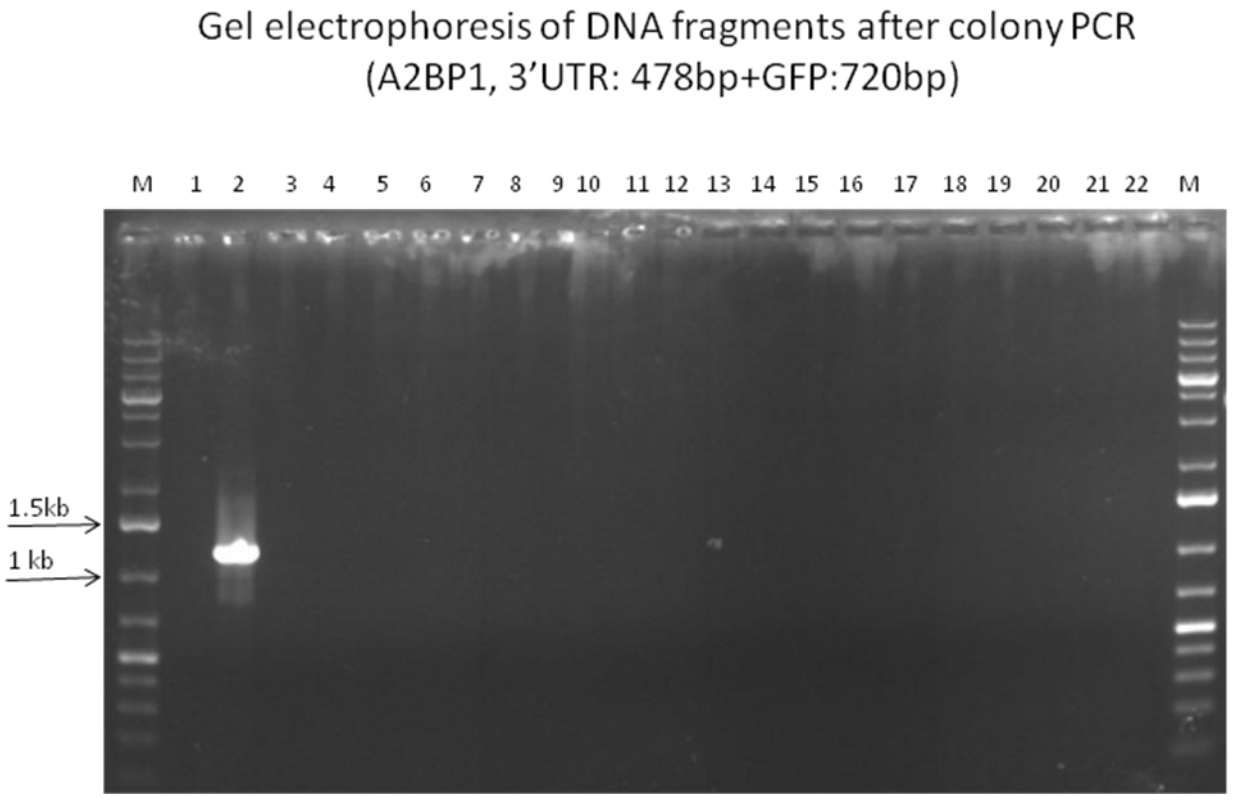


Figure S4. Gel electrophoresis of DNA fragments after colony PCR for pGK1. Each PCR fragment represents one positive recombinant clone

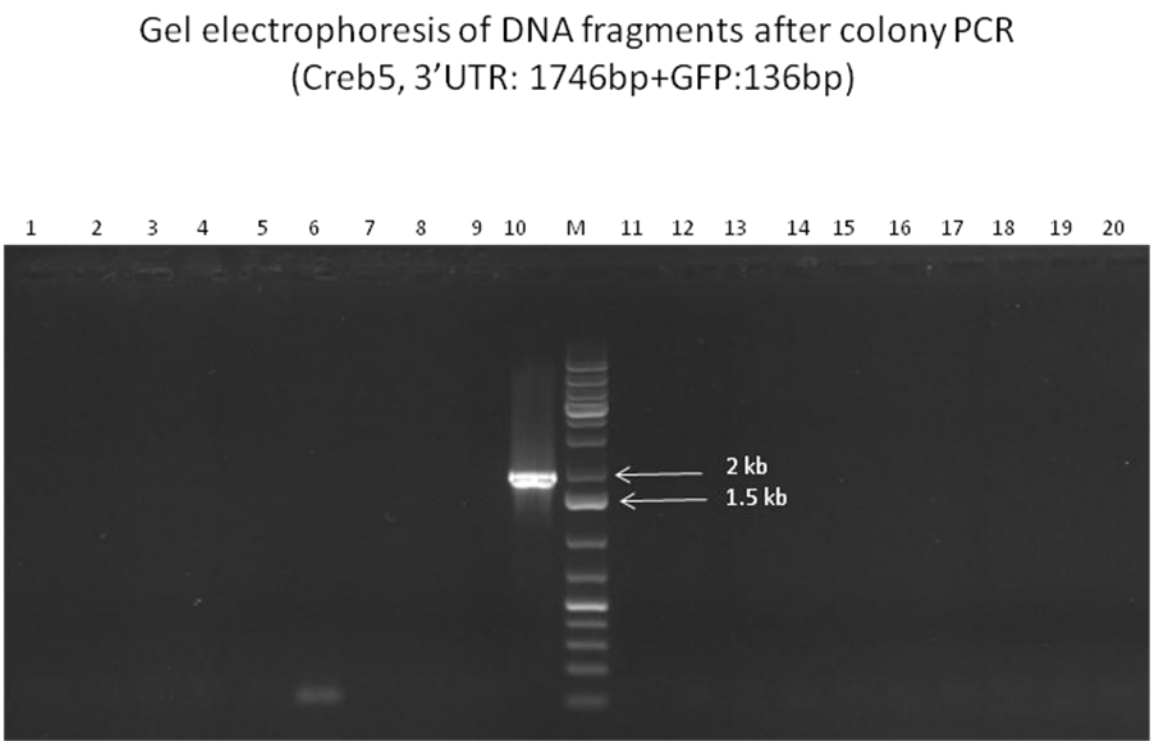


Figure S5. Gel electrophoresis of DNA fragments after colony PCR for pGK2. Each PCR fragment represents one positive recombinant clone

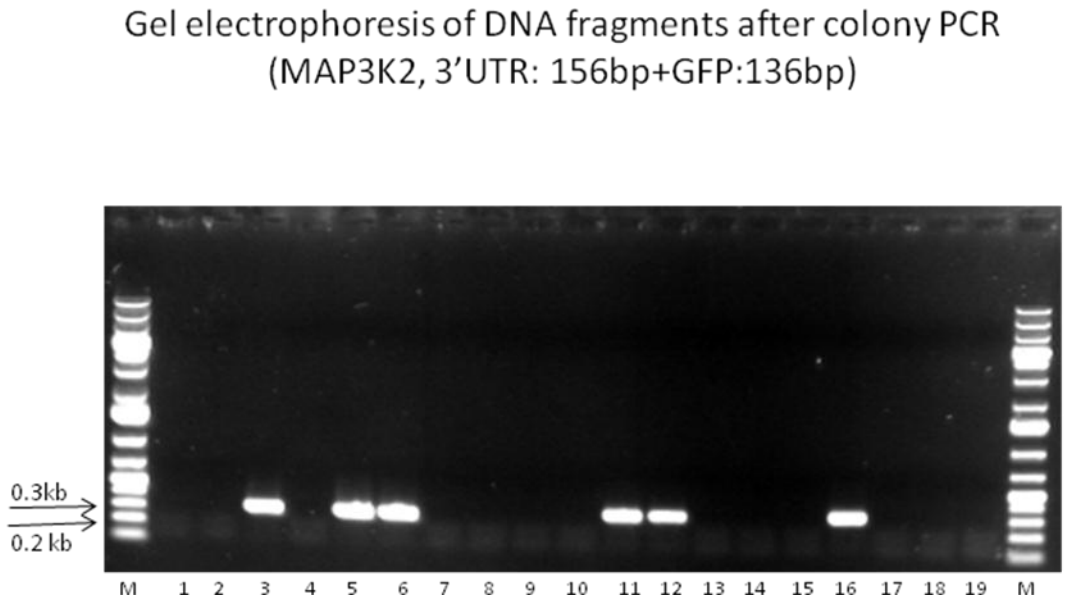


Figure S6. Gel electrophoresis of DNA fragments after colony PCR for pGK6. Each PCR fragment represents one positive recombinant clone

Statistics

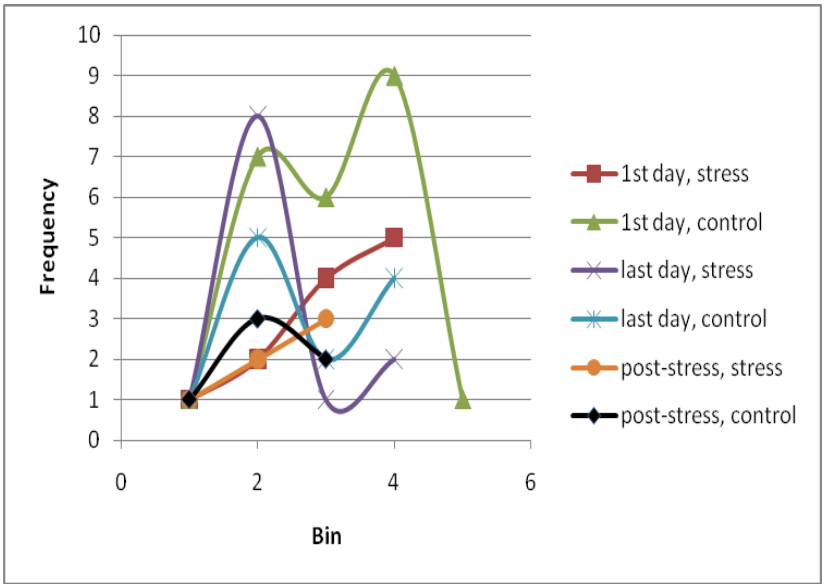


Figure S7. Analysis of frequency distribution of corticosterone in plasma. Some data (represented in red, orange and green) show deviation from normal distribution.

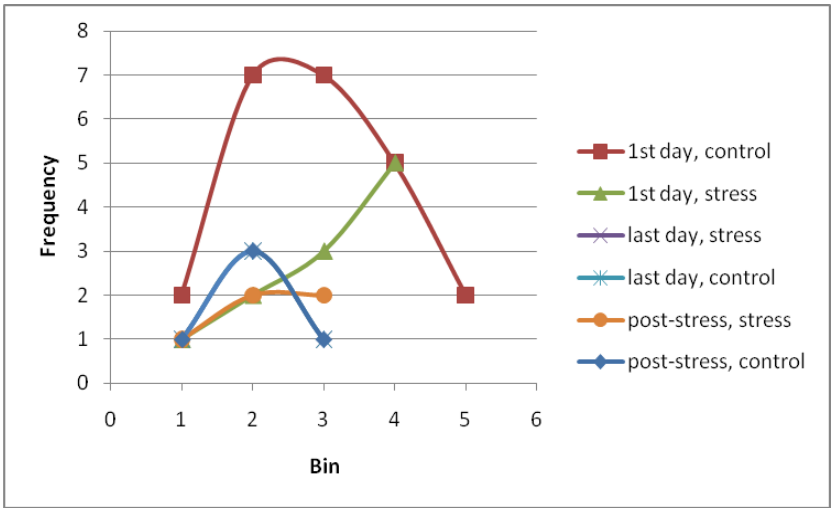


Figure S8. Analysis of frequency distribution of glucose in plasma. Some data points (represented in green and in orange) show deviation from normal distribution.

Luciferase assay

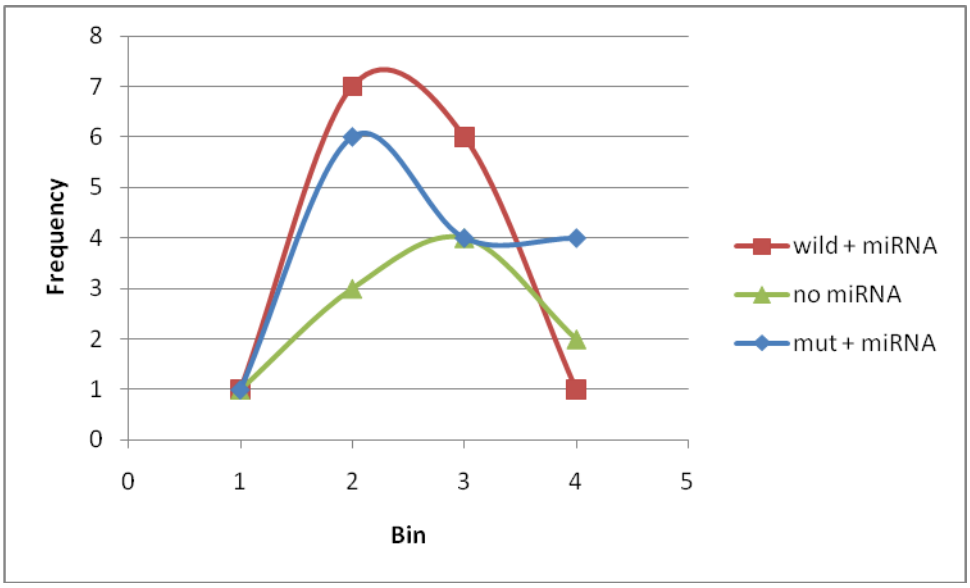


Figure S9. Analysis of frequency distribution of Luciferase activity measurements. Data represented in blue show minor deviation from normal distribution.

Table S1. Statistics for plasma corticosterone

F-Test Two-Sample for Variances

	contol	*stress*
Mean	122.1225	311.3054
Variance	2150.31	5764.027
Observations	6	6
df	5	5
F	0.373057	
P(F<=f) one-tail	0.151528	
F Critical one-tail	0.198007	

t-Test: Two-Sample Assuming Equal Variances

	contol	*stress*
Mean	122.1225	311.3054
Variance	2150.31	5764.027
Observations	6	6

Pooled Variance	3957.168
Hypothesized Mean Difference	0
df	10
t Stat	-5.20895
P(T<=t) one-tail	0.000198
t Critical one-tail	1.812461
P(T<=t) two-tail	0.000396
t Critical two-tail	2.228139

Table S2. Statistics for *Prlr* (2 Weeks)

F-Test Two-Sample for Variances

	control	*stress*
Mean	453.4189601	1610.599
Variance	130331.8563	27983.2
Observations	6	6
df	5	5
F	4.657503549	
P(F<=f) one-tail	0.058319633	
F Critical one-tail	5.050329058	

t-Test: Two-Sample Assuming Equal Variances

	control	*stress*
Mean	453.4189601	1610.599
Variance	130331.8563	27983.2
Observations	6	6
Pooled Variance	79157.52846	
Hypothesized Mean Difference	0	
df	10	
t Stat	-7.123859898	
P(T<=t) one-tail	1.60146E-05	
t Critical one-tail	1.812461102	
P(T<=t) two-tail	3.20291E-05	
t Critical two-tail	2.228138842	

Table S3. Statistics for *Adipoq* (2 weeks)

F-Test Two-Sample for Variances

	control	*stress*
Mean	1172.044393	2802.164
Variance	1340189.63	1611768
Observations	6	6
df	5	5
F	0.831502624	
P(F<=f) one-tail	0.422238436	
F Critical one-tail	0.1980069	

t-Test: Two-Sample Assuming Equal Variances

	control	*stress*
Mean	1172.044393	2802.164
Variance	1340189.63	1611768
Observations	6	6
Pooled Variance	1475979	
Hypothesized Mean Difference	0	
df	10	
	-	
t Stat	2.324020098	
P(T<=t) one-tail	0.021240423	
t Critical one-tail	1.812461102	
P(T<=t) two-tail	0.042480846	
t Critical two-tail	2.228138842	

Table S4. Statistics for *Efnb3* (control)

F-Test Two-Sample for Variances

	2 weeks	*4 weeks*
Mean	445.58	1396.573
Variance	54236.02	279612.1
Observations	6	6
df	5	5
F	0.193969	
P(F<=f) one-tail	0.048055	
F Critical one-tail	0.198007	

t-Test: Two-Sample Assuming Unequal Variances

	2 weeks	*4 weeks*
Mean	445.58	1396.573
Variance	54236.02	279612.1
Observations	6	6
Hypothesized Mean Difference	0	
df	7	
t Stat	-4.03161	
P(T<=t) one-tail	0.002493	
t Critical one-tail	1.894579	
P(T<=t) two-tail	0.004986	
t Critical two-tail	2.364624	

Table S5. Statistics for *Gabra4* (control)

F-Test Two-Sample for Variances

	2 weeks	*4 weeks*
Mean	2315.441903	5961.082
Variance	307081.2376	319452.8
Observations	6	6
df	5	5
F	0.961272659	
P(F<=f) one-tail	0.483242318	
F Critical one-tail	0.1980069	

t-Test: Two-Sample Assuming Equal Variances

	2 weeks	*4 weeks*
Mean	2315.441903	5961.082
Variance	307081.2376	319452.8
Observations	6	6
Pooled Variance	313267.0161	
Hypothesized Mean Difference	0	
df	10	
t Stat	-11.28176569	
P(T<=t) one-tail	2.60423E-07	
t Critical one-tail	1.812461102	

P(T<=t) two-tail	5.20847E-07
t Critical two-tail	2.228138842

Table S6. Statistics for *Gabra4* (stress)

F-Test Two-Sample for Variances

	2 weeks	*4 weeks*
Mean	2315.441903	5961.082
Variance	307081.2376	319452.8
Observations	6	6
df	5	5
F	0.961272659	
P(F<=f) one-tail	0.483242318	
F Critical one-tail	0.1980069	

t-Test: Two-Sample Assuming Equal Variances

	2 weeks	*4 weeks*
Mean	2315.441903	5961.082
Variance	307081.2376	319452.8
Observations	6	6
Pooled Variance	313267.0161	
Hypothesized Mean Difference	0	
df	10	
t Stat	-11.28176569	
P(T<=t) one-tail	2.60423E-07	
t Critical one-tail	1.812461102	
P(T<=t) two-tail	5.20847E-07	
t Critical two-tail	2.228138842	

Table S7. Statistics for Luciferase assay

F-Test Two-Sample for Variances

	wild + miRNA	*no miRNA*
Mean	4.618685143	1.554974
Variance	3.837194944	0.151151
Observations	15	10
df	14	9
F	25.38652116	
P(F<=f) one-tail	1.63368E-05	
F Critical one-tail	3.025472724	

t-Test: Two-Sample Assuming Unequal Variances

	wild + miRNA	*no miRNA*
Mean	4.618685143	1.554974
Variance	3.837194944	0.151151
Observations	15	10
Hypothesized Mean Difference	0	
df	16	
t Stat	5.886006718	
P(T<=t) one-tail	1.14976E-05	
t Critical one-tail	1.745883669	
P(T<=t) two-tail	2.29953E-05	
t Critical two-tail	2.119905285	

Table S8. Disease relationships for genes that changed after two weeks of restraint treatment (2WS vs. 2WC) (Taken from The GeneCards Human Gene Database, Weizmann Institute of Science, with minor modifications; www.genecards.org).

Disease[1]	Articles[2]	PubMed IDs for Articles with Shared Sentences (# sentences)[3]
STRA6		
matthew-wood syndrome	3	18316031 (2), 19760653 (1)
anophthalmia	3	19839040 (2), 19112531 (1)
pagod syndrome	1	19760653 (1)
microphthalmia	1	19112531 (1)
malformation	2	19112531 (1), 18316031 (1)
pda	1	19839040 (1)
TC2N		
abscess; liver	1	19688750 (1)
dystrophy (muscular)	1	17512949 (1)
pigmentosa; retinitis	1	18387594 (1)
mental retardation	1	18387594 (1)
abscess	1	19688750 (1)
shock	1	17407182 (1)
MSX1		
hypodontia	37	10861665 (4), 18788550 (3), 16682758 (3), 16498076 (3)
agenesis	31	17559452 (4), 17973693 (3), 17651126 (3), 8696335 (2)
cleft palate	23	16868654 (4), 12163415 (4), 9870533 (2), 9830770 (2)
cleft lip	19	15379328 (4), 9003904 (2), 17557248 (2), 16868654 (2)
msx1 gene mutation	3	18788550 (1), 17559452 (1), 16687911 (1)
syndrome, wolf-hirschhorn	6	1969845 (3), 14630905 (2), 1685989 (1)
orofacial cleft, nonsyndromic	3	18932005 (3)
ellis-van creveld	3	8882877 (3)
skin; appendage	3	7537773 (3)
craniofacial abnormalities	3	14654219 (1), 12701100 (1), 12163415 (1)
GDF15		
fibrodysplasia ossificans progressiva	45	17967130 (6), 19255227 (5), 17516498 (5), 19896889 (3)
fusion; spine	18	15537446 (3), 15897826 (2), 11716009 (2), 19917933 (1)
heterotopic ossification	26	3263906 (5), 19255227 (2), 19043134 (2), 17967130 (2)

[1] Disease - The name of the disease related to GeneCards gene.
[2] Articles - The number of articles in which both the gene's symbol or description and the disease appear.
[3] PubMed IDs for Articles with Shared Sentences (# sentences) - PubMed IDs of articles in which both the gene symbol and the disease appear in the same sentence, sorted by the number of sentences (shown in parentheses in the column) in which they both appear.

familial pulmonary arterial hypertension	9	20204735 (3), 17989347 (2), 19153267 (1), 18436795 (1)
osteosarcoma	52	8519127 (5), 10939432 (5), 7951046 (4), 11169148 (4)
juvenile polyposis	10	19318548 (2), 15235019 (2), 19622104 (1), 18756288 (1)
fracture non-union	10	19597895 (2), 17669279 (2), 12429748 (2), 8589196 (1)
gastric hamartoma	4	19318548 (4)
ineffective erythropoiesis	8	20182355 (4), 20001631 (1), 19400694 (1), 19036111 (1)
primary pulmonary hypertension	13	12740218 (4), 15192043 (3), 19324947 (1), 16361357 (1)
FOLR1		
ovarian cancer	126	19617914 (7), 19454358 (6), 9426700 (5), 19189636 (5)
deficiency; folate	22	15641086 (5), 9301457 (2), 18355335 (2), 8126512 (1)
epidermoid carcinoma, nasopharyngeal	5	2605182 (1), 2430957 (1), 16478640 (1), 12757377 (1)
neural tube defect	16	18804286 (3), 11102926 (2), 10234517 (2), 9545095 (1)
ovarian tumor	15	18357444 (3), 9099956 (2), 19585555 (2), 18222534 (2)
oral cancer	11	20023317 (1), 19435095 (1), 19088031 (1), 16564654 (1)
mesothelioma	10	12019370 (4), 11174727 (3), 20051956 (2), 19839924 (1)
leukemia l1210	5	9037255 (1), 8765468 (1), 7488246 (1), 2353941 (1)
human mammary carcinoma	3	7682567 (3)
ovarian adenocarcinoma	3	9743473 (2), 7614473 (1)
CITED1		
melanoma	25	11310794 (6), 9683535 (4), 15990678 (4), 11434569 (3)
nevi	7	11310794 (4), 15990678 (2), 9683535 (1)
carcinoma papillary thyroid	6	15072698 (6)
tumor; wilms	4	17710162 (4)
malignant melanocytic lesion	1	15990678 (1)
benign thyroid nodule	1	15072698 (1)
benign nevi	1	15990678 (1)
amelanotic melanoma	1	9721210 (1)
follicular tumor	1	15072698 (1)
tumor initiation	1	17710162 (1)
TCF21		
neck cancer	2	18398044 (1), 16415157 (1)
atrioventricular canal	1	9733105 (1)
growth arrest	1	12493738 (1)
tumor; wilms	1	12012385 (1)
lung cancer	2	18398044 (1), 16415157 (1)
squamous cell carcinoma	1	16415157 (1)
diabetes; type 2	1	19788433 (1)
cancer	3	16415157 (2), 18398044 (1)
PRLR		
breast tumor	44	184919 (3), 177195 (3), 9467590 (2), 7634417 (2)

breast cancer	149	18053149 (6), 18613925 (5), 7639855 (4), 3191209 (4)
hyperprolactinemia	24	18095129 (4), 8159006 (3), 2986084 (3), 11164823 (3)
benign breast tumor	3	6145592 (2), 18779591 (1)
gynecomastia	6	18613925 (5), 11501039 (1)
prolactin deficiency	2	3008583 (1), 10776642 (1)
cholestasis	9	16489923 (2), 16461560 (2), 15527411 (2), 15835542 (1)
cns tumor	4	18095129 (4)
poorly differentiated thyroid carcinoma	2	17525486 (2)
adenomyosis	4	9126864 (3), 10218134 (1)
OTC		
hyperammonemia	156	1549234 (4), 683780 (3), 2372929 (3), 19225137 (3)
otc deficiency	1030	9778587 (7), 9007316 (6), 8857803 (6), 8830175 (6)
aciduria; orotic	20	3732588 (2), 9266387 (1), 9266354 (1), 8938172 (1)
reye syndrome	31	893420 (3), 6823424 (3), 175276 (3), 1782015 (2)
citrullinemia	21	11804205 (3), 18616627 (2), 18562231 (2), 7439194 (1)
carbamoyl phosphate synthetase i deficiency	10	9252147 (2), 7246541 (1), 6724509 (1), 2771513 (1)
hyperargininemia	12	20004862 (4), 7717428 (1), 7439194 (1), 18616627 (1)
encephalopathies	29	7666324 (2), 7499756 (2), 18651132 (2), 18414167 (2)
argininosuccinate lyase deficiency	9	18616627 (2), 18562231 (2), 7717428 (1), 19217439 (1)
deficiency; enzyme	10	980551 (1), 9048915 (1), 8677801 (1), 8464164 (1)
OSMR		
primary cutaneous amyloidosis	11	19690585 (4), 18179886 (4), 19663869 (1), 19375894 (1)
leukemia	10	11602599 (2), 20051625 (1), 18491353 (1), 17979974 (1)
cervical squamous cell carcinoma	3	17516585 (3)
cervical squamous intraepithelial lesion	2	17516585 (2)
prolactinoma	2	11095488 (2)
colon cancer	4	19662090 (4)
inflammation	7	15893881 (4), 18641356 (2), 18179886 (1)
adenoma	3	11095488 (3)
colorectal polyp	1	19223499 (1)
colorectal cancer	4	19223499 (3), 19662090 (1)
ITGB6		
squamous cell carcinoma	9	12076695 (3), 12917446 (2), 19528463 (1), 15033492 (1)
colon cancer	6	11992542 (2), 9705874 (1), 18162155 (1), 15668738 (1)
emphysema	4	20358620 (2), 12634787 (2)
oral cancer	3	12917446 (3)
tumor progression	3	12917446 (3)
inflammation	6	9761761 (2), 8666675 (1), 18385522 (1), 17200187 (1)
pocket formation	1	18385522 (1)

renal fibrosis	1	17200187 (1)
alport syndrome	1	17200187 (1)
pancreatic ductal adenocarcinoma	1	15330800 (1)
CORIN		
cardiac hypertrophy	6	18669922 (2), 18716601 (1), 16054049 (1), 16054020 (1)
failure heart	10	20061521 (4), 15191894 (3), 15155264 (2), 16054020 (1)
dfnb24	1	17918732 (1)
hypertension	12	18716601 (3), 18669922 (2), 16216958 (2), 19131349 (1)
tapvr	2	11082206 (2)
sclc	4	14678991 (4)
dysplasia; epithelial	2	7952341 (2)
cardiovascular disease	4	17996891 (1), 17485366 (1), 16054020 (1), 11082206 (1)
hypertensive heart disease	1	15637153 (1)
myocardial necrosis	1	15659305 (1)
CLDN3		
ovarian cancer	30	19567823 (4), 15905176 (4), 12855632 (4), 19208807 (3)
papillary serous carcinoma	9	17326053 (4), 19426958 (2), 18313739 (2), 16725184 (1)
ovarian tumor	6	19208807 (2), 18057528 (1), 16287068 (1), 15899825 (1)
atypical hyperplasia	4	17291259 (4)
increased; permeability	4	16798726 (1), 16275920 (1), 12907427 (1), 12570983 (1)
cerebral hypoperfusion	3	18782597 (3)
endometrial cancer	6	18313739 (5), 17326053 (1)
effusion	5	18439941 (5)
carcinosarcoma	3	17545541 (3)
acidosis metabolic chronic	2	17383680 (1), 16675746 (1)
ADIPOQ		
diabetes; type 2	825	19584347 (7), 17980008 (6), 17010797 (6), 16358955 (6)
insulin resistance	1748	19755479 (6), 19732605 (6), 19486470 (6), 19302182 (6)
obese	2059	18253163 (7), 16421343 (7), 16222059 (7), 16092047 (7)
ovary; polycystic (syndrome)	227	15579189 (11), 19261627 (8), 17562334 (8), 17071536 (8)
tumor necrosis	557	19462491 (3), 17059516 (3), 20136458 (2), 19539174 (2)
insulin sensitivity	1102	16286515 (8), 19107128 (7), 16571847 (7), 16373895 (7)
metabolic syndrome	775	19214966 (11), 16964963 (8), 16755284 (8), 19409756 (7)
fatty liver	143	15793855 (6), 15579183 (5), 20022856 (4), 19958641 (4)
hyperinsulinemia	161	19820029 (4), 17893258 (4), 19751297 (3), 16414018 (3)
nonalcoholic steatohepatitis	77	15718867 (9), 16231364 (6), 19502655 (4), 18923878 (4)

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