

DISS. ETH Nr. 21577

**STUDY ON THE IMPACT OF EARLY CHRONIC STRESS ON  
BEHAVIOR ACROSS GENERATIONS IN MICE**

ABHANDLUNG

zur Erlangung des Titels

DOKTORIN DER WISSENSCHAFTEN

der

ETH ZÜRICH

vorgelegt von

KATHARINA GAPP

Mag. Rer nat., Universität Wien

geboren am 14. Juni 1984

*Staatsbürgerin der Schweiz und Österreichs*

Angenommen auf Antrag von

Isabelle Mansuy

Carmen Sandi

Minoo Rassoulzadegan

2014



"I have enjoyed peaches and apricots more since I have known that they were first cultivated in China in the early days of the Han dynasty."

Bertrand Russell

## **Abstract**

Epigenetic regulation of gene expression is mediated via a complex ensemble of mechanisms that comprise DNA methylation, histone post-translational modifications and non-coding RNAs. It allows cells to commit to a specific identity and to fine-tune their functions in response to intrinsic and extrinsic signals. On a large scale, it enables organisms to persistently adapt to environmental conditions. Epidemiological studies suggest that organisms can transmit acquired information to subsequent generations. Similarly, rodent models indicate that environmental challenges, such as early life stress, not only affect the exposed individuals persistently but can also impact their offspring and grand offspring. Early life stress is a major risk factor for the development of neuropsychiatric disease in humans. It can alter the function of the hypothalamic-pituitary-axis, the major stress-regulatory system in the body. It is believed that such alterations are mediated by epigenetics, thus providing a mean of persistently modulating subsequent stress responses. An altered stress response induced by chronic stress can be maladaptive or advantageous, depending on the environmental context. It can compromise flexibility and promote habit behavior in a non-stressful surrounding. Under stressful conditions, it might however have the opposite effect and thus prepare animals for an adverse environment. Exposure to an enriched environment has been suggested to counteract the development of maladaptive behaviors. But it is unknown whether it can further prevent the inheritance of maladaptation to early life stress and clear mechanisms for such an epigenetic inheritance are still poorly understood and hotly debated. Small RNAs have been proposed as potential mediators between genes and environment, but their putative implication in the inheritance of the effect of early life stress has not been studied yet.

The current thesis investigates the effects of unpredictable maternal separation combined with unpredictable maternal stress on behavioral responses, gene expression of stress hormone receptors and their epigenetic regulation in the male line offspring in the mouse. Further, it explores the potential of exposure to an enriched environment to counteract the transmission of the effect of early life stress on behavior. Finally, it studies the processes responsible for the inheritance of the effects of early trauma across generations.

The present study shows that early life stress alters the expression of the mineralocorticoid receptor, one of the main stress hormone receptors in the brain. It provides evidence that the mineralocorticoid receptor is subject to epigenetic regulation via histone post-translational modifications and DNA methylation in response to paternal stress. This results in decreased expression and is associated with altered behavioral flexibility across 3 generations. In addition, we show that environmental enrichment can prevent the transmission of some of the behavioral alterations induced by early life stress. Lastly, we observed that early life stress can alter small RNAs in serum, brain and sperm which are associated with changes in glucose metabolism. Injection of the altered small sperm RNAs of mice exposed to early life stress into fertilized oocytes was sufficient to induce behavioral alterations in the resulting offspring, mimicking those present in the natural offspring of stressed fathers.

The data presented in this thesis demonstrate that early life stress can prime the offspring for a specific environmental condition, in part through altered epigenetic regulation of the mineralocorticoid receptor in the hippocampus. It proposes EE as a means to counteract the transmission of maladaptive traits. Moreover, this work provides a mechanism for the transmission of acquired traits, by showing that altered sperm RNAs contribute to the transmission of the effect of early life stress on metabolic and behavioral changes.

## Zusammenfassung

Epigenetische Regulierung der Genexpression beruht auf einem komplexen Ensemble an Mechanismen, wie DNA-Methylierung, Histon-post-translationale Modifikationen und nicht-kodierenden RNAs. Sie definiert die jeweilige Identität einer Zelle oder eines Gewebes und präzisiert Zellfunktionen in Abhängigkeit von Umwelteinflüssen. Auf höherer Ebene, ermöglicht sie dem Organismus sich langfristig an Änderungen in der Umwelt anzupassen. Epidemiologische Studien lassen darauf schließen, dass Organismen erworbene Eigenschaften oder Informationen an die Folgegenerationen weitergegeben können. Auch Studien in Nagetieren belegen langanhaltende Effekte von Umwelteinflüssen, wie z.B. Stress in der frühen Kindheit, nicht nur auf die den Bedingungen ausgesetzten Individuen sondern auch auf deren Nachkommen. Im Menschen stellt Stress in der frühen Kindheit, einen großen Risikofaktor für eine spätere Erkrankung an neuropsychiatrischen Krankheiten dar. Stress beeinflusst die Funktion der Hypothalamus-Hypophysen-Nebennierenrinden-Achse, was, so glaubt man, auf epigenetische Prozesse zurückzuführen ist. Eine durch Stress in der frühen Kindheit ausgelöste Veränderung der Stressreakтивität kann sich negativ oder positiv auswirken, in Abhängigkeit von den zukünftig vorhandenen Umweltgegebenheiten. Das kann, in einer wenig stressvollen Umgebung, eine kompromittierte Verhaltensflexibilität und erhöhtes rigides Gewohnheitsverhalten zur Folge haben während in einer anderen Umwelt gegensätzliche Effekte zu tragen kommen können. Somit könnte ein Organismen auf ein bestimmtes Milieu vorbereitet werden. Ein stimulierendes, ausgestaltetes Umfeld soll den durch Stress in der frühen Kindheit ausgelösten Fehlanpassungen entgegenwirken. Ob es auch das Potential hat generationenübergreifend diese zu korrigieren ist derzeit nicht bekannt. Genaue Mechanismen für solch eine epigenetische Vererbung sind ebenfalls ausstehend. Es wird vermutet, dass kleine nicht-codierende RNAs an dem Wechselspiel zwischen Genen und Umwelt beteiligt sind. Ihre vermeintliche Rolle in der Vererbung der Verhaltenseffekte von frühkindheitlichem Stress wurde noch nicht erforscht.

Diese Doktorarbeit untersucht die Effekte von unvorhersehbarer Mutter-Kind Trennung kombiniert mit mütterlichem Stress auf Verhalten, Genexpression von Stresshormonrezeptoren und deren epigenetische Regulierung in den Nachkommen. Darüber hinaus beschäftigt sie sich mit dem Potential einer stimulierenden, ausgestalteten Umwelt, der Vererbung von Verhaltenseffekten ausgelöst durch Stress in der frühen Kindheit entgegenzuwirken. Letztendlich, wendet sie sich den Prozessen

zu, welche einer Vererbung der Effekte des frühkindheitlichen Stresses zugrunde liegen könnten.

Die hier vorliegende Studie zeigt, dass Stress in der frühen Kindheit die Genexpression des Mineralocorticoidrezeptors, eines der Hauptstresshormonrezeporen im Hirn, beeinträchtigt. Sie weißt eine epigenetische Regulierung dieses Rezeptors in Abhängigkeit von Umwelteinflüssen nach, sowohl auf der Ebene von DNA-methylierung wie auch von Histon-post-transkriptionalen Modifikationen. Stress in der frühen Kindheit führt zu einer reduzierten Expression und geht einher mit erhöhter generationenübergreifender Verhaltensflexibilität. Gleichzeitig fanden wir heraus, dass ein stimulierendes angereichertes Umfeld einer Vererbung der Effekte von frühkindheitlichen Stress entgegenarbeitet. Schließlich beschreiben wir unsere Beobachtungen über die Auswirkung von Umwelteinflüssen auf kleine nicht-kodierende RNAs in Serum, Hirn und Sperma und auf den Glucosemetabolismus. Eine Injektion der veränderten Sperma-RNAs von frühkindheitlich gestressten Tieren war ausreichend um Verhaltensveränderungen in den daraus resultierenden Nachkommen zu induzieren, welche denen der natürlichen Nachkommen sehr ähnlich waren.

Diese Thesis zeigt, dass Stress in der frühen Kindheit von Mäusen deren Nachkommen für eine spezifische Umwelt vorbereiten kann, teilweise bedingt durch eine veränderte epigenetische Regulierung des Mineralocorticoidrezeptors im Hippocampus. Sie schlägt eine Intervention vor um einer Vererbung von Fehlanpassungen vorzubeugen. Letztendlich liefert die vorliegende Arbeit einen Mechanismus welcher zumindest teilweise für die epigenetische Vererbung von erworbenen Eigenschaften verantwortlich gemacht werden kann, indem sie belegt, dass veränderte kleine nicht-kodierende RNAs im Sperma zur Weitergabe der Verhaltens- und metabolischen Effekte von frühkindheitlichen Stress über Generationen beitragen.

## **Table Of Contents**

<b>1. OVERVIEW &amp; INTRODUCTION .....</b>	<b>10</b>
1.1. STRESS AND ITS IMPACT ON BEHAVIOR.....	10
1.2. EARLY LIFE STRESS AND EPIGENETICS.....	10
1.3. EPIGENETIC TRANSGENERATIONAL INHERITANCE .....	11
1.4. THE MSUS MODEL .....	12
1.5. AIM OF THE THESIS .....	12
<b>2. EARLY LIFE EPIGENETIC PROGRAMMING AND TRANSMISSION OF ACQUIRED TRAITS IN MAMMALS .....</b>	<b>14</b>
2.1. SUMMARY .....	15
2.2. INTRODUCTION .....	16
2.3. THE BRAIN IS SUSCEPTIBLE TO STRESS DURING CRITICAL PERIODS IN LIFE .....	18
2.4. THE CHARACTERISTICS OF STRESS EXPOSURE DETERMINE THE CONSEQUENCES ON BRAIN AND BEHAVIOR.....	19
2.5. THE EFFECTS OF ENVIRONMENTAL EXPOSURE CAN BE PASSED ONTO THE FOLLOWING GENERATION(S) .....	21
2.6. POTENTIAL ROUTES OF TRANSMISSION OF ACQUIRED TRAITS ACROSS GENERATIONS .....	23
2.7. CONCLUSIONS .....	35
2.8. ACKNOWLEDGMENTS .....	35
2.9. AUTHORS` CONTRIBUTION .....	36
<b>3. EARLY LIFE STRESS IMPROVES BEHAVIORAL FLEXIBILITY IN THE OFFSPRING AND INVOLVES EPIGENETIC REGULATION .....</b>	<b>37</b>
3.1. ABSTRACT.....	38
3.2. INTRODUCTION .....	39
3.3. MATERIALS AND METHODS .....	39
3.4. RESULTS.....	46
3.5. DISCUSSION .....	51
3.6. ACKNOWLEDGMENTS .....	53
3.7. AUTHORS` CONTRIBUTION .....	53
3.8. SUPPLEMENTARY MATERIAL.....	54
<b>4. ENVIRONMENTAL ENRICHMENT COUNTERACTS THE TRANS-GENERATIONAL TRANSMISSION OF THE EFFECTS OF EARLY LIFE STRESS ON MOOD AND ANXIETY-RELATED BEHAVIOR .....</b>	<b>66</b>
4.1. ABSTRACT.....	67
4.2. INTRODUCTION .....	67
4.3. METHODS .....	68
4.4. RESULTS.....	72

4.5. DISCUSSION .....	76
4.6. ACKNOWLEDGEMENTS .....	80
4.7. AUTHORS' CONTRIBUTION .....	80
<b>5. IMPLICATION OF SPERM RNAs IN THE INHERITANCE OF THE EFFECTS OF EARLY TRAUMATIC STRESS IN MICE .....</b>	<b>81</b>
5.1. SUMMARY .....	82
5.2. MAIN TEXT .....	83
5.3. ACKNOWLEDGEMENTS .....	92
5.4. AUTHORS' CONTRIBUTION .....	93
5.5. SUPPLEMENTARY MATERIAL.....	94
5.6. SUPPLEMENTARY METHODS .....	108
<b>6. DISCUSSION .....</b>	<b>114</b>
6.1. SIGNIFICANCE .....	114
6.2. LIMITATIONS .....	118
6.3. OUTLOOK.....	120
<b>7. REFERENCES .....</b>	<b>122</b>
<b>8. APPENDIX 1: EARLY LIFE STRESS PROMOTES STRESS RESILIENCE IN THE OFFSPRING.....</b>	<b>140</b>
8.1. ABSTRACT.....	141
8.2. INTRODUCTION: .....	142
8.3. MATERIALS AND METHODS .....	142
8.4. RESULTS.....	146
8.5. DISCUSSION .....	152
8.6. ACKNOWLEDGEMENTS .....	154
8.7. AUTHORS` CONTRIBUTION.....	154
<b>9. APPENDIX 2: EPIGENETIC REGULATION IN NEURODEVELOPMENT AND NEURODEGENERATIVE DISEASES .....</b>	<b>155</b>
9.1. ABSTRACT.....	156
9.2. INTRODUCTION .....	157
9.3. EPIGENETICS IN BRAIN DEVELOPMENT AND AGING .....	157
9.4. CNS DISORDERS WITH AN EPIGENETIC BASIS .....	164
9.5. CONCLUDING REMARKS.....	170
9.6. ACKNOWLEDGEMENTS .....	171
9.7. AUTHORS` CONTRIBUTION.....	171
<b>10. APPENDIX 3: ACKNOWLEDGEMENTS.....</b>	<b>172</b>
<b>11. APPENDIX 4: CURRICULUM VITAE .....</b>	<b>174</b>

# **1. Overview & Introduction**

## **1.1. Stress and its impact on behavior**

The exposure to severe or long-lasting stressful experiences has detrimental effects on a wide range of physiological and psychological functions in humans, primates, and rodents (1). In humans, traumatically stressful experiences early in life, including physical or sexual abuse, are particularly detrimental and constitute major risk factors for the development of emotional and cognitive disorders in adulthood, ranging from major depression to attention and anxiety disorders (2). Early trauma alters general stress sensitivity throughout life, which in turn predisposes to psychiatric disorders (3, 4). Whether predisposed individuals will actually develop disease symptoms often, critically depends on the environment they encounter later in life (5). In rodents, early life stress has similarly dramatic and long-lasting effects on behavior. It alters emotional and stress-responses, and can induce depressive-like behavior (2). Therefore, rodent models, which allow for high experimental control, are extremely valuable to study the mechanisms by which early stress alters behavior. These mechanisms are only beginning to be revealed and are postulated to involve epigenetic processes since they are induced by environmental factors and in many cases persist for a lifetime (6).

## **1.2. Early Life Stress and Epigenetics**

Epigenetics refers to mechanisms that modify the chromatin to stably and dynamically control gene expression without altering the DNA sequence (7). The underlying mechanisms are not fully understood but include, DNA methylation and hydroxymethylation, RNA methylation, post-transcriptional modifications of protein histones and non-coding RNAs(8) among others. Such processes can hinder or facilitate the binding of transcription factors and recruit or block the transcriptional machinery (9). Because the DNA sequence is highly stable throughout the lifespan of an organism (except in the case of exposure to carcinogens, UV, mutagens, etc.), epigenetic modifications provide a level of genetic plasticity which allows an organism to adapt and respond to environmental factors by changing the pattern of gene expression (10). Using rodent models, it has been demonstrated that epigenetic programming in response to early life experience can cause stable, lifelong alterations in gene expression and can thereby affect behavior in adulthood (6). A well-described example is maternal investment, where naturally occurring differences in the quality of maternal care during the first week of life lead to lasting changes in DNA methylation

that affect gene expression in adulthood (11). The fact that differences in the quality of maternal care can have a such long-lasting impact throughout life suggests that more dramatic experiences might produce even stronger and more persistent changes. For example, chronic severe stressful experience induced by repeated social defeat leads to depressive-like symptoms in mice and induces epigenetic changes involving an increase in repressive histone post translational modifications and reduced expression of neurotrophic factors (12).

### **1.3. Epigenetic Transgenerational Inheritance**

Several lines of research have provided evidence that the epigenetic code can be changed in response to environmental factors (13-17), and that the central nervous system seems to have adopted epigenetic processes to add a layer of plasticity to information processing and storage (18). Despite this plasticity, epigenetic changes can also be highly stable across the lifespan of an organism, and in some cases, they can even be transmitted to subsequent generations (19-25). In the past few years, several studies in rodents demonstrated transgenerational effects of chronic stress (26-28), insufficient maternal care(29) and drug exposure(25). Further, rodent studies (30) (31, 32)and humans (33, 34) have shown transgenerational effects on metabolism of gestational diet. Further, in certain conditions, early life environmental enrichment has enhancing effects on cognitive performance that are long lasting and can be transmitted across generations (35, 36). An elegant study in plants (radish) and animals (water flea) has shown the evolutionary adaptive value of the ability of parents to transmit acquired defensive mechanisms to their offspring, by demonstrating a decreased vulnerability to predators in the following generations (37). These behaviors persisted for several generations, but gradually disappeared if the predator was not present. The transient nature of these adaptations highlights the fact that they cannot represent alterations in DNA sequence, but rather specific changes in gene expression, presumably brought about by epigenetic mechanisms (38). Further experimental evidence supporting epigenetic inheritance indicates that changes induced by environmental toxins can be transmitted across several generations and increase the risk of infertility and cancer (10, 21, 39-43). Recently, several studies carried out in *Drosophila* and *C. elegans* reported on transgenerational inheritance of environmentally induced traits and provided firm evidence for small RNAs to be involved in the mechanism of transmission (44-46). The propagation of some environmentally induced epigenetic and behavioral alterations across generations, combined with the fact that early life experience can alter epigenetic marks as well as

behavior (6), opens the intriguing possibility that adverse events early in life may affect not only on the animal exposed, but also subsequent generations. This possibility is clinically relevant because in humans, heritability is known to be a major risk factor for emotional and mood disorders (47). Thus, epigenetic changes could account for some of the heritable disease risk associated with psychiatric disorders such as depression (48). Rodent models of early life stress are ideally suited to test this possibility, as stress effects across several generations can be examined in relatively short time, and because high experimental control allows dissociating genetic from environmental factors.

#### **1.4. The MSUS model**

In an attempt to better understand the mechanisms that underlie the impact of early stress on behavior across generations, the Mansuy lab has developed a novel mouse model of early chronic stress in mice (22). This model is based on unpredictable maternal separation combined with maternal stress (MSUS), a manipulation that reproduces stressful experiences in early life. When adult, the stressed mice develop depressive-like behaviors and display altered risk assessment and sociability (49). These behavioral alterations are strong and strikingly persistent, as they are transmitted paternally (49, 50) and maternally to the following F2 and F3 generation, despite the fact that the offspring is never exposed to stress and raised under standard facility conditions (non-stressed F2 offspring). Therefore, in the MSUS model, life history of the father determines the behavior phenotype of the offspring. The behavioral alterations are accompanied by an aberrant profile of DNA methylation in the promoter of several genes, not only in the germ line of the males subjected to MSUS but also in the brain of their offspring (22). These results suggest a potential epigenetic mode of inheritance of the detrimental effect of early stress on behavior across generations. Therefore, the MSUS paradigm is an intriguing model to study the mechanisms underlying the heritability of stress-induced molecular changes and its impact on behavior across generations.

#### **1.5. Aim of the thesis**

This thesis aimed to assess (1) the effect of early life adversity on behavioral flexibility and its potential underlying molecular mechanisms across generations in mice, (2) the potential of environmental enrichment to prevent the transmission of the effect of early

life stress on behavior, and (3) whether small RNAs may underlie the transgenerational epigenetic transmission of the effects of early life stress.

In the first chapter we summarize the literature on the effect of a variety of environmental factors, such as stress, nutrition, endocrine toxins and drugs, during different stages of development on long-term epigenetic regulation and behavior in mammals. Further, we discuss their transmission across generations and the potential mechanisms involved in this process.

The second chapter presents our findings regarding the effect of early life stress on behavioral flexibility across generations. It also describes the associated alterations in epigenetic regulation of gene expression of the mineralocorticoid receptor.

In the third chapter we provide evidence that the exposure to an enriched environment following early life stress can prevent the transmission of stress-induced behavioral changes across generations

The fourth chapter suggests that small non-coding RNAs in sperm can carry information from one generation to the next. Specifically, we provide evidence that miRNAs and piRNAs are altered in the sperm of animals exposed to early life stress and contribute to the transmission of the effect of early life adversity on depressive-like and anxiety behaviors, metabolism and altered protein expression.

Finally, we summarize and reconcile the findings of the different chapters. We interpret the overall results from an evolutionary perspective and discuss pitfalls of the studies and potential future directions.

## **2. Early life epigenetic programming and transmission of acquired traits in mammals**

Katharina Gapp<sup>1</sup>, Lukas von Ziegler<sup>1</sup> and Isabelle M Mansuy\*<sup>1</sup>

<sup>1</sup> Brain Research Institute, University of Zürich and Swiss Federal Institute of Technology, Winterthurerstrasse 190, Zürich, CH-8057, Switzerland, CH-8057 Zürich

\*Corresponding author, Email: mansuy@hifo.uzh.ch

*How and when can environmental factors influence traits and their transgenerational inheritance?*

**In review at Bioessays**

**Keywords:** epigenetic inheritance, early life stress, acquired traits

## **2.1. Summary**

Environmental factors can have a long-lasting influence on an individual's physiology and behavior, and can induce adaptive responses to changing living conditions. Yet in other cases they lead to pathological behaviors. Epidemiological studies have shown that changes induced by the environment can be expressed not only by the individuals directly exposed, but also by their offspring across several generations. Epigenetic mechanisms have been proposed to underlie such persistent and transmissible effects. Here, we review some of the current knowledge on critical developmental stages during which such changes can occur. We discuss current evidence for transgenerational effects on body functions and behavioral responses induced by prenatal and postnatal factors, and potential epigenetic mechanisms. We also discuss the need for a careful evaluation of their evolutionary importance with respect to health and diseases, and possible directions for future research in the field.

Abbreviations: CpG, cytosine-guanine; DNAme, DNA methylation; HPTMs, histone posttranslational modifications; mRNA, messenger RNA; PGC, primordial germ cell; K, lysine; sncRNAs, small non-coding RNAs.

## **2.2. Introduction**

### **2.2.1. Changes in the environment induce behavioral adaptation**

The ability to perceive and appraise surrounding environments and properly respond to these environments is an important feature for living organisms (51). It allows them to suitably react to stimuli and increase their chance to survive and reproduce (52). Keeping a memory of such adaptive response is essential to efficiently cope with similar conditions when encountered later in life (53). Such adaptation is usually beneficial and helps adjusting to surrounding conditions, but it can also be maladaptive when environmental requirements dramatically change and no longer match the initially adapted behaviors (54). Such mismatch between an adapted response and the encountered milieu can lead to inappropriate and pathological behaviors, and increase disease predisposition (55). Thus, although an inherited trait may be thought to be naturally selected when beneficial, a maladapted trait can also be retained across generations (56). The biological mechanisms underlying adaptive behaviors are complex and involve activity-dependent changes in gene expression in several neural circuits and brain regions (55). Because they are modulated by the environment and are not genetically encoded, some of these changes are mediated by non-genomic (epigenetic) processes (57, 58)

### **2.2.2. The epigenetic code controls genome activity**

One of the main functions of epigenetic processes is to remodel the chromatin and activate or silence genes. The chromatin comprises the DNA helix wrapping around octamers of histone proteins to form nucleosomes (59). It can be structurally remodeled by covalent modification of the DNA and histones, in particular, DNA methylation (DNAm) and histone posttranslational modifications (HPTMs). The

ensemble of these modifications constitute an epigenetic code that alters gene activity but without changing genomic sequences themselves (7). In mammals, DNAm is a biochemical process that involves the covalent addition of a methyl group to cytosines in DNA, preferentially in CpG (cytosine-guanine) dinucleotides (60). Likewise, HPTMs are covalent modifications occurring on protein histones that include acetylation, methylation, phosphorylation, ubiquitylation, etc (61). Further to these mechanisms, non-coding RNAs (ncRNAs) also contribute to gene regulation. NcRNAs are small or long RNAs that, unlike messenger RNAs (mRNAs), are thought not to be translated into proteins but act as regulators of gene expression. They promote mRNA degradation and down-regulate protein translation (62). They can also act to guide epigenetic components to specific DNA sequences (63). The ensemble of the modifications brought about by DNAm and HPTMs (and to some extent ncRNAs) establishes an epigenetic profile that is dynamically regulated at each gene. These marks locally modify the electrical properties of chromatin, alter its conformation and regulate its accessibility to the transcriptional machinery (64). This ultimately modifies gene transcription in a spatially- and temporarily-regulated manner in response to internal and external factors (65, 66).

### **2.2.3. Epigenetic processes contribute to the transmission of acquired traits**

Because gene programming or re-programming by chromatin remodeling is persistent and can be maintained across life(11, 29, 58), it has the potential to be carried over to the following generation if present in the germline (21). Transmission of adaptive traits is a very important biological process that has a potentially significant impact on evolution (67). Such transmission has the advantage to provide an optimized response to an environment previously encountered by the parental generation. If the encountered environments do not match between generations an inherited trait can also be maladaptive (56). Mechanistically however, although it clearly does not involve any genetic change, transgenerational inheritance is difficult to be intuitively

associated with epigenetic modifications because some epigenetic marks, in particular DNA methylation, are known to be globally erased during germ cell development and in the early zygote. However, the fact that some genes i.e. imprinted genes and other specific loci (68), can maintain or re-instate their epigenetic profile despite reprogramming and remain imprinted in the progeny strongly suggests that epigenetic profiles can persist across generations. Here, we review some of the current evidence showing that the acquisition of traits induced by environmental factors can occur during different phases of development, that such acquired information can be transferred across generations, and that it potentially involves epigenetic mechanisms. We focus on traits induced by environmental changes in early life and their consequences on behavioral responses later in life and across generations.

### **2.3. The brain is susceptible to stress during critical periods in life**

The influence of environmental factors on the body and its underlying epigenetic mechanisms have been studied in relation to brain functions. In the brain, the (re)programming of epigenetic marks by environmental factors depends on cellular responses to intrinsic and extrinsic signals (69). It contributes to various brain processes and functions such as memory formation (13), drug addiction (14) and stress responses (15). In some cases, these marks are transient and dynamically regulated (16, 17), while in others, they can persist and be perpetuated (22). The strength and persistence of epigenetic changes strongly depend on the developmental stage and time of their establishment. The prenatal period (70), early childhood (71) and adolescence (72) are critical temporal windows for the influence of environmental conditions in mammals. During these developmental phases, the brain goes through extensive growth (73) and remodeling (65), and is particularly sensitive to external conditions and to interference (74). Environments involving stress are particularly detrimental. In humans, stressful conditions experienced by a pregnant woman

increase the incidence of neurodevelopmental disorders such as schizophrenia and autism spectrum disorders in the child (75-77). Likewise, in laboratory animals like rodents, gestational stress applied to the mother alters stress sensitivity, behavior, morphology, and gene expression in the resulting offspring (78, 79). Environmental conditions in early postnatal life also strongly influence development, and can increase predisposition to psychiatric disorders later in life in humans (80). This is also the case in animals, in which the level of maternal care is particularly critical. Maternal nursing is directly associated with the formation of proper behavioral responses later in life, and to the susceptibility to stress-induced disorders in adulthood. This link was shown to implicate epigenetic mechanisms of gene regulation, in particular, changes in DNAm of a regulatory region of the glucocorticoid receptor (*NR3C1*) in the hippocampus (11). Adolescence is another critical window during which stress exposure can have detrimental consequences on mental health later in life. In humans, maltreatment during adolescence can induce antisocial behaviors in young adults (81). In rodents, hyper-activation of the hypothalamic-pituitary-adrenal (HPA) axis due to stress during this period also alters behavioral responses and elicits multiple symptoms including increased aggression and antisocial behaviors (82, 83).

## **2.4. The characteristics of stress exposure determine the consequences on brain and behavior**

The impact and long-term consequences of stress exposure are known to depend on the type, severity and duration of the stressor(s). Stressors include a variety of environmental conditions such as psychological challenge and nutritional restriction.

### **2.4.1. Altered maternal care perturbs adult behaviors**

The quality of the social and parental environment early in life is a critical determinant of the proper development of an individual. In humans, prolonged separation from the

mother and maternal neglect predispose an individual to behavioral deviance such as drug abuse later in life, in part by altering reward pathways (84). In rodent models, while predictable maternal separation (subjected at the same time daily) often has no lasting behavioral effects in the offspring due to compensatory maternal behaviors (85), unpredictable and fragmented stress strongly compromises maternal sensory signals and triggers persistent cognitive and emotional dysfunctions later in life (86). In mice, unpredictable maternal separation combined with unpredictable maternal stress was shown to lead to a wide range of behavioral symptoms including depressive-like behaviors, social withdrawal, impaired social recognition and reduced risk assessment (22, 49, 50). Interestingly at the same time, this manipulation also increases behavioral flexibility and makes the animals more reactive in challenging situations (our own unpublished observations), suggesting that unpredictable stress in early life can also provide some benefit later in life. However, in most cases, psychological stress acts as a negative factor, and it is only with favorable conditions such as exposure to enriched conditions that beneficial effects may be observed (87). Interestingly, long-lasting effects of living conditions early in life have also been reported to be sex-dependent. In some cases, although both females and males can be affected, the extent of behavioral alterations such as depressive-like behaviors induced by repeated exposure to forced swim stress, can depend on gender (88).

#### **2.4.2. Malnutrition applies stress to the organism**

One of the first reported examples of the consequences of fetal under-nutrition in human is the effect of diet restriction in young age. A large-scale study in a Dutch cohort subjected to winter hunger at the end of World War II (Hunger Winter Families Study) showed that individuals born from mothers undernourished during pregnancy have altered epigenetic marks (17, 18). A differentially-methylated region of the imprinted gene *IGF2* was shown to be hypomethylated in blood of individuals born to these women up to 60 years after the hunger period (33). Many of these individuals

suffer from metabolic alterations (36, 37, 53) and have a higher prevalence of psychiatric dysfunctions including higher incidents of schizophrenia, and uni- and bipolar disorder (8, 9, 21). In rodent models, stress evoked by malnutrition also alters behaviors and impacts brain functions. Maternal high-fat diet during gestation increases anxiety and alters serotonin level in the hippocampus in mice (89). It also reduces corticosterone and increases the level of its receptors in the amygdala in the offspring (7). Likewise in rats, direct exposure to high-fat diet for a long period (8 weeks) also increases anxiety and corticosterone levels (90). However in contrast to long exposure, short exposure (1 week) to high-fat diet has an opposite effect and is anxiolytic (91).

## **2.5. The effects of environmental exposure can be passed onto the following generation(s)**

Numerous epidemiological and clinical studies in human have underscored a strong heritable component in mood disorders for instance, in major depressive disorder (MDD) (92), post traumatic stress disorder (93) and associated externalizing and internalizing traits (94). However up to now, the heritability of these disorders could not be attributed only to genetic factors, and genes influencing such complex diseases have been proposed to act either as low penetrance common variants, or rare, highly penetrant inherited mutations. In the case of MDD for instance, only approximately 40% of the risk was determined to be genetic (73), with the remaining 60% remaining as “missing heritability”. Such missing heritability was postulated to be accounted for by environmental factors. Such factors may affect not only the exposed individuals but also their offspring, and thereby potentially impact several generations. This suggests that epigenetic factors brought about by the environment, likely underlie some of the inheritance of complex diseases (47, 95, 96). This hypothesis is strengthened by a

recent epidemiological study that showed that paternal obesity leads to *IGF2* hypomethylation in newborns (23), suggesting that paternal malnutrition has a transgenerational influence on *IGF2*. Since *IGF2* is a hormone that plays an essential role in promoting growth during gestation and is necessary for cognitive processes throughout life (3, 57), it will be interesting to see whether the alterations in *IGF2* persist into adulthood and contributes to psychiatric disease risk.

Animal models have proven useful to study this question and the underlying mechanisms. For instance, exposure to chronic traumatic stress during the first 2 weeks of life persistently alters behavioral responses across several generations in mice. Unpredictable maternal separation combined with unpredictable maternal stress in young mouse pups causes depressive-like behaviors and deficits in novelty response, risk assessment and social behaviors in adulthood (49, 50, 97). These behavioral symptoms are transmitted to the following generation through both females and males (up to 3 generations for males) and are independent of maternal care. They are associated with alterations in DNAme in several stress-related genes, in the adult brain and sperm in first and second-generation animals, and with altered expression of these genes in the brain. Likewise in rat, adolescent stress has an impact across generations. The offspring of stressed rat dams have increased anxiety but also display better sociability and improved avoidance learning (36). Interestingly, exposure to an enriched environment before gestation also has an effect on the offspring but that is opposite to that after stress exposure. The offspring of enriched dams show sex-dependent differences in anxiety levels and reduced avoidance learning when compared to the offspring of stressed dams (36). Further in juvenile mice (postnatal day 15 to 30), exposure to enriched conditions can rescue a deficit in synaptic plasticity in adulthood. It reverses a defect in hippocampal long-term potentiation (LTP), a form of synaptic plasticity linked to memory processes, in the exposed animals but also in the adolescent progeny of these animals (35).

Thus, traits acquired by environmental exposure have the potential to be transmitted across generations. There are however various potential routes of transmission involved in such transmission.

## **2.6. Potential routes of transmission of acquired traits across generations**

Traits acquired by environmental exposure can be maintained and transferred from one generation to the next through different routes. Some routes depend on the presence of the initial triggering conditions, which are needed at each generation to reinstate the traits. Such routes are based on behavioral and social transfer. Other routes involve more stable mechanisms that become independent of the initial trigger, and reflect a molecular transfer implicating germ cells.

### **2.6.1. Behavioral and social transfer**

Many traits acquired following exposure to environmental factors are transmitted from one generation to the next through behavioral and social interactions in early or adult life. For instance, in mammals, the quality and level of maternal care in early postnatal life have a strong influence on the progeny's development, and determine their physiological and behavioral responses later in life. In rats, maternal behaviors in mothers condition maternal behaviors in the female offspring. Thus, female rats providing insufficient maternal care to their pups give rise to female offspring that become themselves poor mothers. Mechanistically, such behavioral transfer is associated with broad epigenetic changes across the genome that affect multiple genes (98). Further in male rats, exposure to non-social stressful experiences in youth increases aggression towards females in adulthood, an effect also observed in the offspring of these males. Such transfer involves depressive behaviors of dams subjected to mistreatment by their mate, and aggressive behaviors in the male

offspring (99).

### 2.6.2. Molecular transfer

Pioneering studies on plants and invertebrates have provided initial insight into the mechanisms potentially involved in epigenetic inheritance. They demonstrated that non-coding RNAs can act as carriers of information across generations and contribute to the transfer of acquired traits (44, 45, 100, 101). In mammals however, the mechanisms involved remain not fully determined. These mechanisms are thought to be multiple and depend on the developmental stage of induction. They determine the penetrance of the effects and their perpetuation across subsequent generations. In this respect, the inheritance of environmentally-induced traits can be considered truly transgenerational and epigenetic only if they do not need the trigger at each generation, and if they are observed in individuals of the third generation, whose founder germ cells have not been exposed to the trigger (102). The expression of the traits in these individuals is an indication that epigenetic mechanisms in the germline are at play (103). Studying these mechanisms in mammals is however difficult because germ cells are not easy to collect and to analyze. Further ideally, both maternal and paternal lines (matrilines and patrilines respectively) need to be examined. However, patrilines have the advantages to exclude maternal care confounds and possible social and/or behavioral transfer, and to prevent interference by somatic components of oocytes and by the *in utero* environment. Sperm cells are also more abundant than oocytes and easier to use for molecular analyses. However, since true epigenetic inheritance also occurs in matrilines (50), findings in male germ cells need to be validated in females. The following section discusses the importance of the developmental stages for the induction of persistent traits and presents various observations of acquired traits transmission. Although the findings are based on rodent studies, a mechanistic translation to humans is reasonable given that the time

window of susceptibility of germ cells relative to birth (pre versus postnatal exposure) is comparable in mice and humans.

#### ***2.6.2.1. How epigenetic changes are transmitted across generations critically depends on the time of induction***

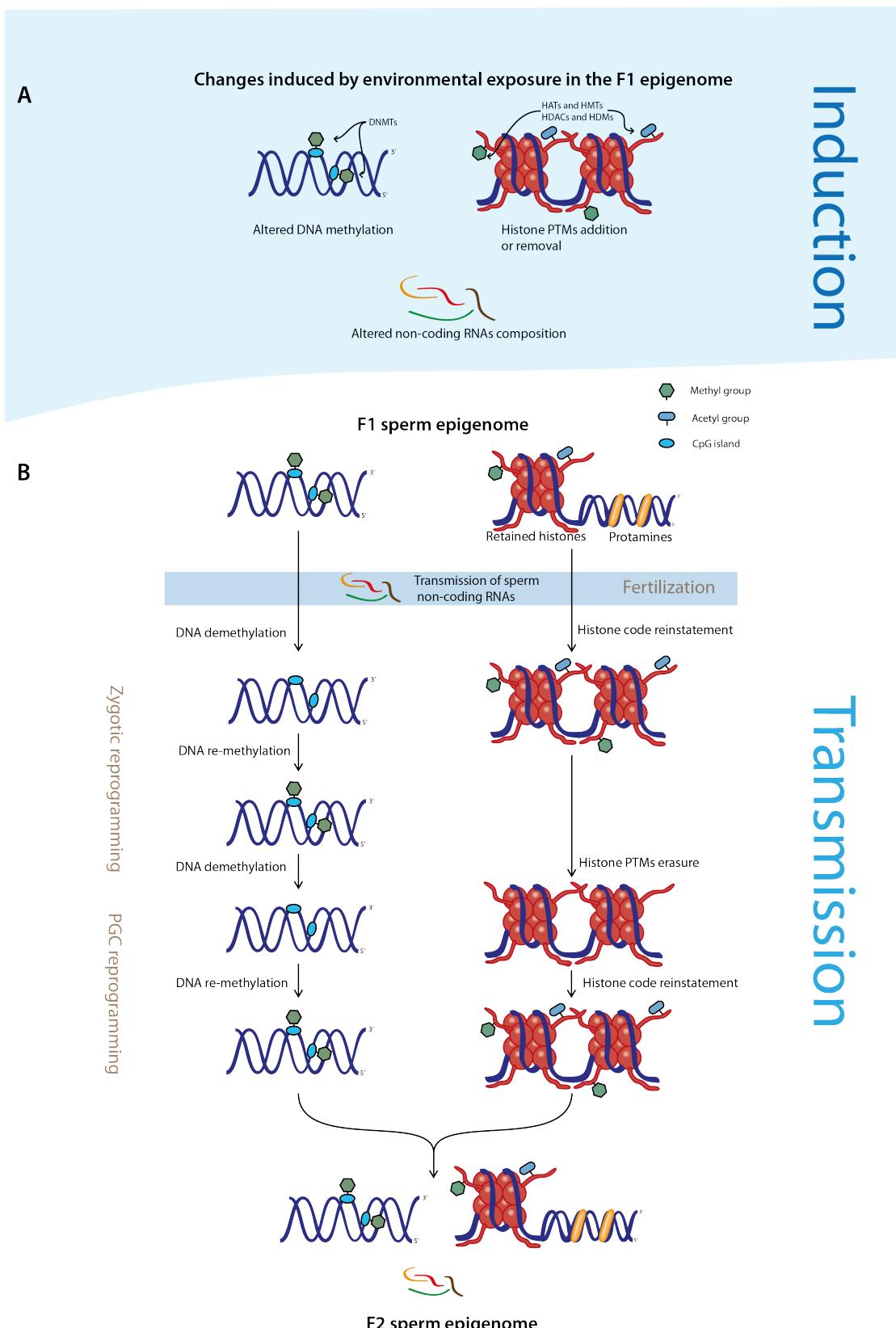
The mere observation that an environmental condition induces epigenetic changes in the germline and specific traits in the subsequent generation does not guarantee true epigenetic inheritance. For true epigenetic inheritance to occur, epigenetic changes need to be persistent and if not persistent themselves (for instance, a change in HPTMs in germ cells may only be transient), they need to be relayed by more stable/different marks. The occurrence and persistence of epigenetic changes is determined by the timing of the environmental exposure (Fig. 1). Although in theory, epigenetic changes can occur throughout life, they are more likely to happen during early stages of development, in particular during epigenetic (re)programming of germ cells or the embryo when the genome is in a susceptible state (104).

##### *Zygotic epigenetic reprogramming*

Epigenetic reprogramming is a complex cascade of molecular events occurring in early development and during which epigenetic marks involving DNAme and HPTMs are dynamically established by successive waves of marking and erasure (105, 106). In the early zygote, the maternal and paternal genome coming from gametes have a different epigenetic profile, but then undergo zygotic reprogramming. Immediately after fertilization and till the morula stage at preimplantation, DNAme marks are globally erased. In the female pronucleus, passive demethylation occurs upon consecutive cell divisions, while there is active demethylation in the male pronucleus (72). DNA erasure affects genes globally but spares a few of them, in particular imprinted genes but also genes expressed in the male germline (68), repeat-associated IAP retrotransposons (107) and genes in heterochromatin within and

around centromeres (108). Further, soon after fertilization in the male pronucleus, protamines (histone-like proteins partially replacing histones during spermatogenesis) are exchanged with maternally-inherited histones (109), then lysine (Lys, K) acetylation followed by methylation occur on specific residues i.e. on K5 and K12 on H4 (105). Some maternal HPTMs such as K9 and K27 methylation which is established during oocyte growth, are however maintained (105) and therefore constitute an epigenetic memory.

# Induction



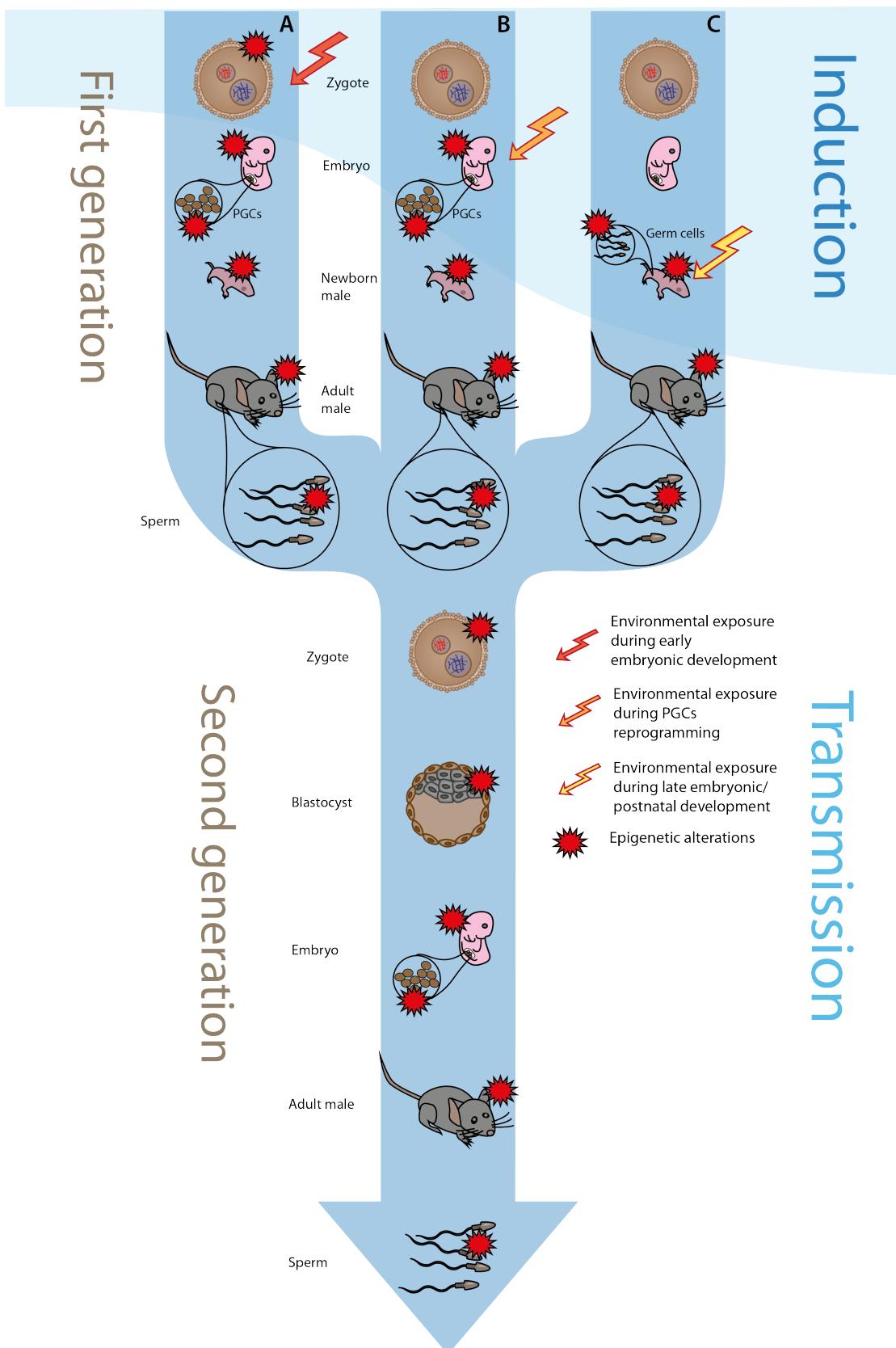
# Transmission

**Figure 1.** Induction and transmission of the effects of environmental exposure on the epigenome. A) Induction: Environmental factors can alter DNA methylation, histone post-translational modifications (PTMs) and the composition of non-coding RNAs in

animals exposed either directly during early life or adulthood (F1) or through their mother (F0) during pregnancy/gestation. Multiple molecular modifiers can contribute to alterations in DNA methylation and histone PTMs including DNMTs, which induce DNA methylation, and HATs, HMTs, HDACs and HDMs, which acetylate/methylate or desacetylate/demethylate histones respectively. DNA methylation and histone PTMs locally alter the properties of chromatin, such as structure and charge, and thereby lead to changes in gene activity. B) Transmission: Some epigenetic marks might be maintained in germ cells during DNA and histone reprogramming from F1 to F2, and contribute to epigenetic inheritance. To be transmitted, DNAme marks have to escape global DNAme erasure during fertilization or be correctly reinstated after erasure. Transmission of histone PTMs also requires reinstatement of the histone code, which in germ cells, is compromised by the replacement of most histones by protamines during sperm maturation(110). It therefore requires the retention of selected histones, or a correct reinstatement of PTMs in the zygote after fertilization. Sperm non-coding RNAs that are delivered to the oocyte at fertilization might contribute to these processes (28, 94, 111). For further inheritance to F3, epigenetic alterations need to resist reprogramming in the F2 epigenome (in PGCs). Abbreviations: PTMs, post translational modifications; DNMTs, DNA methyltransferases; HATs, histone acetyltransferases; HMTs, histone methyltransferases; HDACs, histone deacetylases; HDMs, histone demethylases.

### *Epigenetic reprogramming of primordial germ cells (PGCs)*

Another wave of reprogramming takes place in primordial germ cells (PGCs), which are germ cells precursors in the early embryo. During this wave, DNAm and HPTMs i.e. H3K9me2 from germ cells are globally erased across the genome (106, 112). But again, although most DNAm marks are erased, some are maintained in specific loci for instance, in genes containing or near repeat-associated IAP elements and in subtelomeric regions (113). Imprinting is then established (114, 115) to keep a parent-specific epigenetic mark and determine whether the maternal or paternal allele is expressed (116). Further, at a later stage of postnatal maturation in sperm, H4 variants also become hyperacetylated to allow nucleosome dissociation. Then, most histones are substituted by protamines to provide a tighter packaging of the DNA (110). However, some histones and their HPTMs, for instance H3K4me3 and H3K27me3, can be retained at loci containing developmental genes (117), and therefore provide another means to maintain epigenetic marks. Protamines in adult sperm can also carry multiple PTMs (our own unpublished observations) suggesting the possibility that histone-protamine transition or protamine PTMs may contribute to information transfer from one generation to the next. Functionally, the successive waves of epigenetic reprogramming are paralleled by differential regulation of gene expression in the embryo (118). Transcription of both female and male genomes is increased at 2- and 4-cell stages but the male genome is more permissive to transcription during later zygotic stages(119). Germ cells chromatin is therefore highly responsive during epigenetic reprogramming and is in a configuration susceptible to epigenetic alterations. The extent and persistence of alterations depend on the time point of environmental exposure relative to epigenetic reprogramming. Depending on whether a perturbation by environmental factors occurs shortly after fertilization or later in development or in adulthood, its impact and likelihood of transmission are different. Several time-dependent scenarios for patriline inheritance can therefore be envisaged (Fig. 2) and used to distinguish potential different mechanisms.



**Figure 2.** Induction and transmission of environmental exposure during development.

A) Environmental exposure during early embryonic development, for instance before

E10 in rodents, is likely to affect all somatic cells including future PGCs in the embryo. Such induction is the most effective when occurring in the zygote through to the blastocyst (between E0 and E3.5) in the first generation since the chromatin is under reprogramming during this stage and is therefore more susceptible to alterations (72, 85, 120, 121). B) Environmental exposure between E10 and E13 may perturb proper PGCs reprogramming, and epigenetic marks that resist zygotic reprogramming after fertilization - not as extensive as PGCs reprogramming (107, 108)- are present in the individuals derived from these germ cells. C) Environmental exposure during late embryogenesis and postnatal development can also induce heritable epigenetic changes in germ cells although germ cells at this stage of development are less susceptible to interference. In A-C, true transgenerational transmission requires that epigenetic changes persist through both germ cell and zygotic reprogramming.

Abbreviations: E, embryonic day; PGCs, primordial germ cells.

#### ***2.6.2.2. Evidence for the involvement of different epigenetic mechanisms in the molecular transmission of acquired traits***

Several studies have examined the effects of environmental exposure during embryogenesis, early postnatal development, adolescence or adulthood across generations in experimental animals (see Table 1 and 2). Although DNAme, histone PTMs and non-coding RNAs have been proposed as potential transgenerational carriers of information, DNAme has been explored most extensively. Environmental exposure impacting imprinted genes is particularly interesting since the mechanisms operating to protect these genes from reprogramming (93, 122) may be recruited for transgenerational inheritance of acquired traits. Studies on vinclozolin or stress exposure have indeed shown that imprinted genes can be affected (20), suggesting increased susceptibility of these genes to environmental changes. However, no difference in the degree of susceptibility could be detected for exposure after imprint

establishment in PGCs (24). Future studies should determine whether these genes might dispose on a higher susceptibility during imprinting in PGCs.

Indeed, no substantial reprogramming takes place postnatally in the male germline, suggesting that the germline epigenome should be less susceptible to interference later in development. However, epigenetic marks continue to be established during postnatal life in the male germline (123), making them a target of interference. Studies in our lab showed that imprinted genes can still be affected (22). It has been suggested that environmental exposure could render epigenetic modifications of non-imprinted genes, imprint-like (68, 107, 108, 113) and thereby enable their transmission.

Mechanistically, the inheritance of traits acquired after birth may also involve different mechanisms than during embryogenesis. HPTMs and ncRNAs are likely candidates to contribute to transmission. Histones and protamines can both carry PTMs, and ncRNAs are present in sperm cells and may be altered by external factors. Indeed, there is initial evidence that small ncRNAs may be involved in the transmission of stress-induced traits. For instance, exposure to chronic stress for 6 weeks during puberty or adulthood alters a pool of miRNAs in sperm, and reduces HPA axis responsiveness in the offspring. Unpredictable traumatic stress in early postnatal life also alters miRNAs content in mouse sperm, and has effects across generations that are associated with sperm RNAs (our own unpublished observations). How sperm RNAs are involved in the transmission of the stress effects however still needs to be determined (28). These results corroborate previous data showing that sperm RNAs can underlie the transmission of a genetically-induced phenotype (111).

Effects of prenatal environmental exposure on subsequent generations									
Study	Model organism	Environmental exposure	Timing	Behavioral alterations	Physiological alterations	Epigenetic mechanism involved	Breeding modality	Generations investigated	True epigenetic inheritance
Anway et al. 2005 <sup>39</sup>	rat	Vinclozolin	E8-E15		DNAme in testis of F1 males; decreased spermatogenic capacity	DNAme	interbreeding of descendants	1,2,3,4	yes
Skinner et al. 2008 <sup>40</sup>	rat	Vinclozolin	E8-14	sex-specific anxiety behavior in F3	sex-specific alteration in hippocampal gene expression		outbred; inter breeding of descendants	3	yes
Guerrero-Bosagna et al. 2010 <sup>19</sup>	rat	Vinclozolin	E8-E14		DNAme at the promoter of most genes that contain a specific consensus sequence in the germline	DNAme	outbred; inter breeding of descendants	3	yes
Stouder et al. 2010 <sup>20</sup>	rat	Vinclozolin	E10-E18		altered DNAme at imprinted genes in tail, sperm, liver, skeletal muscle; decreased motile sperm in F1	DNAme	male line	1,2,3	yes
Morgan et al. 2011 <sup>27</sup>	mice	chronic stress	E1-E7	increased stress sensitivity in F1, increased depressive-like behavior and decreased anogenital distance in F2 males	hormonal regulation, dysmasculination of neurodevelopmental gene expression and miRNA expression in F2		male line	1,2	no
Crews et al. 2012 <sup>41</sup>	rat	Vinclozolin	E8-14	altered anxiety in response to stress in F3	overall altered metabolic activity in brain, altered testosterone level in response to stress, altered hippocampal gene expression		non-littermate inter breeding of descendants	3	yes
Wolstenholme et al. 2012 <sup>42</sup>	rat	Bisphenol A	gestation	altered sociability down to F4	estrogen receptor, oxytocin and vasopressin expression		outbred; for F1: crossfostering to control mothers, for F2: brother-sister pairing	1,2,3,4	yes
Guerrero-Bosagna et al. 2012 <sup>43</sup>	mouse	Vinclozolin	E7-E13		spermatogenic cell defects, testicle, prostate and kidney abnormalities, polycystic ovarian disease only in outbred descendants		outbred and inbred strain, no littermate inbreeding	3	yes
Skinner et al. 2013 <sup>21</sup>	rat	Vinclozolin	E8-E14		germline	DNAme	outbred strain, inter breeding of descendants	3	yes

**Table 1: Fetal environmental exposures can have an effect on subsequent generation**

Effects of postnatal environmental exposure on subsequent generations									
Study	Model organism	Environmental exposure	Timing	Behavioral alterations	Physiological alterations	Epigenetic mechanism involved	Breeding modality	Generations investigated	True epigenetic inheritance
Arai et al. 2009 <sup>39</sup>	mouse	environmental enrichment w/wo mutant background	postnatal week 2-4	increased brain plasticity, increased learning			female line with crossfostering for mutant background	1,2	no
Roth et al. 2009 <sup>29</sup>	rat	aversive maternal care	PND1-PND7		altered BDNF gene methylation and expression in prefrontal cortex		female line with crossfostering	1,2	no
Franklin et al. 2010 <sup>22</sup>	mouse	unpredictable maternal separation combined with unpredictable maternal stress	PND1-PND14	depressive-like behavior, altered approach avoidance behavior	altered gene expression and DNAm at the promoter of stress related genes and MeCP2 in F2 hippocampus, and F1 and F2 sperm	DNAm	male line	1,2,3,	yes
Franklin et al. 2011 <sup>49</sup>	mouse	unpredictable maternal separation combined with unpredictable maternal stress	PND1-PND14	alterations in sociability in F2 and F3, in social recognition in F1, F2 and F3, altered response to social defeat in F2	altered 5HT1AR binding and serotonin level in the brain		male line	1,2,3	yes
Weiss et al. 2011 <sup>50</sup>	mouse	unpredictable maternal separation alone or combined with unpredictable maternal stress	PND1-PND14	depressive-like behavior in F1, altered approach avoidance behavior in F1 and F2	altered CRFR2 binding in the brain in F1		female line with crossfostering	1,2	no
Dietz et al. 2011 <sup>26</sup>	mouse	chronic social defeat	days in adulthood	depressive-like and anxiety behavior	sex-specific increase in corticosterone, decrease in vascular endothelial growth factor in F2		male line & IVF using naive oocytes	1,2	no
Leshem et al. 2012 <sup>39</sup>	rat	environmental enrichment and or mild stress	stress: PND27-29; EE: PND21 - PND60	avoidance learning, anxiety, sex-specific effect on acoustic startle test, decreased social interaction in males			female line	2	no
Rodgers et al. 2013 <sup>28</sup>	mouse	chronic stress	6 weeks during adolescence		reduced HPA axis responsiveness	altered miRNAs in sperm	male line	2	no
Vassoler et al. 2013 <sup>25</sup>	rat	cocaine self-administration	60 days in adulthood	sex-specific cocaine resistance	increased BDNF gene expression and acetylation in the medial prefrontal cortex, altered H3 acetylation in the germline of fathers	retained histone PTMs	male line	1,2	no

**Table 2. Post-natal environmental exposures can have an effect on subsequent generation**

## **2.7. Conclusions**

Environmental exposure can have long lasting effects on brain and behavior that can persist over several generations. The mechanisms underlying such transgenerational persistence involve epigenetic mechanisms, which enable the stable transmission of the molecular basis of acquired traits. Despite some reports of molecular transfer or true transgenerational inheritance of acquired traits, these mechanisms remain mostly unknown. This is in part due to their complexity and the difficulty to study them in animal models, and even more so in humans. Thus, the analysis of these mechanisms first requires the establishment of robust, consistent and reliably transmitted phenotypic traits in a model, and the timely and targeted measurement of epigenetic marks in the right tissue or cells, and on the right genes or loci. Further a proper timing of the environmental exposure is also essential. So far, most studies have used models with wide timing of exposure (several days to several weeks) and a single stage as read-out of epigenetic alterations and have therefore not allowed determine the most critical time window of induction, and the time course of epigenetic changes. Moreover, in addition to DNAm, HPTMs and sncRNAs, other non-genomic processes such as 5-hydroxy-DNAm, RNA methylation, long non-coding RNAs would also be interesting to examine. Clearly, such processes and mechanisms likely intertwine with genetic factors, studies considering genome-epigenome interactions will be required. The use of novel techniques and methodological approaches such as high-throughput epigenetic screening and molecular imaging are expected to help gain better understanding on these mechanisms, and on their functional and evolutionary impact (124).

## **2.8. Acknowledgments**

The lab of IMM is funded by the University Zürich, the Swiss Federal Institute of Technology, the Swiss National Science Foundation, Roche, The National Center of

Competence in Research “Neural Plasticity and Repair”, the Austrian Doc Fforte program.

## **2.9. Authors` contribution**

K.G. wrote the manuscript. L.vZ. prepared figures and helped with figure legends. I.M supervised the writing.

### **3. Early life stress improves behavioral flexibility in the offspring and involves epigenetic regulation**

Katharina Gapp<sup>1</sup>, Saray Soldado-Magraner<sup>1</sup>, María Alvarez-Sánchez<sup>2</sup>, Johannes Bohacek<sup>1</sup>, Huan Shu<sup>1</sup>, Gregoire Vernaz<sup>1</sup>, Tamara B. Franklin<sup>3</sup>, David Wolfer<sup>4</sup> and Isabelle M. Mansuy<sup>1\*</sup>

<sup>1</sup>Brain Research Institute, Medical Faculty of the University of Zürich and Department of Health Sciences and Technology of the Swiss Federal Institute of Technology, Neuroscience Center Zürich

<sup>2</sup>Institute of Veterinary Physiology, Vetsuisse Faculty, Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich

<sup>3</sup>EMBL Monterotondo, Italy

<sup>4</sup>Zurich Center for Integrative Human Physiology (ZIHP), Institute of Anatomy of the University of Zürich, and Institute for Human Movement Sciences of the Swiss Federal Institute of Technology Zürich.

\*Corresponding author: IMM, mansuy@hifo.uzh.ch

**Reviewed at Nature Neuroscience. To be submitted at Biological Psychiatry.**

### **3.1. Abstract**

**Background:** Traumatic experiences in childhood can alter behavioral responses and increase the risk for psychopathologies across life, not only in the exposed individuals but also in the progeny. Such experiences can however also be beneficial in some conditions, and can facilitate the appraisal of adverse environments later in life.

**Methodology:** Mice were exposed to unpredictable maternal separation combined with unpredictable maternal stress throughout their first two weeks of life to assess the effect of early life adversity on behavioral flexibility and epigenetic regulation of the mineralocorticoid receptor in the offspring.

**Principal Findings:** Here, we show that postnatal maltreatment in male mice favors goal-directed behaviors and behavioral flexibility in the offspring when adult. This behavioral effect is associated with epigenetic changes involving histone posttranslational modifications at the mineralocorticoid receptor (MR) gene and decreased MR expression in the hippocampus. Mimicking these changes pharmacologically *in vivo* reproduces the behavioral phenotype.

**Conclusions:** Our findings underscore the importance of epigenetic regulation for the impact of early experiences on adult behavior across generations.

### **3.2. Introduction**

Adverse environmental conditions in early life can alter stress responses (125), and affect behavioral and cognitive functions in adulthood. Further, they can also not only affect the individuals directly exposed to stress but have as well, an impact on the progeny (126, 127). In human, traumatic stress during childhood is known to increase the risk for psychiatric conditions such as attention deficit hyperactivity disorder (ADHD), bipolar depression and antisocial behaviors across families (128, 129). However, although in many cases, stress has negative consequences, it can also be beneficial in some cases and confer some advantage later in life. In human, mild-to-moderate stressors experienced in childhood have a “stress inoculating” effect, and favor adaptive responses and resilience upon exposure to other stressors later in life (130, 131). Likewise in animals, chronic exposure to adverse conditions in postnatal life can lower stress reactivity and improve cognitive abilities like spatial and reversal learning in adulthood (64, 132). Whether such beneficial effect can also be observed in the offspring has however not been investigated and remains unclear.

Using a model of unpredictable maternal separation and maternal stress (MSUS) in the mouse (22) we examined the possibility that traumatic stress in postnatal life can have behavioral benefits in the offspring. We found that MSUS increased performance of goal directed behavior and reversal learning, indicating that early life stress indeed leads to decreased avoidance behavior and an increase in behavioral flexibility in aversive environments. These behavioral changes were accompanied by altered epigenetic regulation of the mineralocorticoid receptor (MR) leading to decreased expression and could be mimicked by pharmacologically blockage or epigenetic alteration of the MR. Our finding newly highlight the epigenetic regulation of stress receptors and provide evidence for their critical involvement in a transgenerational priming effect in response to early life stress.

### **3.3. Materials and Methods**

#### **3.3.1. Animals**

Adult (3-6 months old) C57BL/6 mice (Janvier SAS, Berthevin, France) were used. The mice were maintained under a reverse light-dark cycle (light off at 8:00 am) in a temperature- and humidity-controlled facility with food and water *ad libitum*. Behavioral testing was carried out during the first five hours (h) of the animals’ active cycle, which corresponds to daily corticosterone peak in mice(133). For IntelliCages, behavior was automatically recorded for 1 h twice a day (11-12:00 and 16-17:00) in every animal through implanted transponders. F2 females were used for behavioral experiments in

the Intellicage (DDT and BST) because males cannot be tested due to strong behavioral confounds by hierarchical fights resulting from group housing. Females and males were used for molecular experiments. All experiments were conducted in accordance with guidelines and regulations of the cantonal veterinary office of Zürich. License Nr 55-2012.

### **3.3.2. Treatment**

For unpredictable maternal separation combined with unpredictable maternal stress (MSUS), dams and litters were subjected to 3 h proximal separation daily from postnatal day 1 to 14. During separation, dams were exposed to either 20-minutes restraint stress or 5-minutes forced swim stress unpredictably. Control animals were left undisturbed except for a cage change once a week. Once weaned (postnatal day 21), pups were reared in social groups (4-5 mice/cage) composed of animals of the same sex subjected to the same treatment but from different mothers to avoid litter effects. To obtain second-generation animals, F1 control and MSUS males were mated to naïve primiparous C57BL/6 adult females, thereby circumventing potential transmission via altered behavior (127) or altered lactation (125) in female mothers that were directly exposed to stress during early life. Males were removed immediately after mating. A total of 30 females (F0, 15 Controls and 15 MSUS) were used to generate F1 animals and 54 F1 males (27 Controls and 27 MSUS) were used to produce F2 animals in 3 independent experiments, yielding 14 control and 20 MSUS litters (see Supplementary Figure 1 for representation of the MSUS paradigm).

### **3.3.3. Behavioral testing**

Behavioral testing was carried out by experimenters blind to treatment. Behaviors were monitored by direct observation and by automated videotracking (Viewpoint, France). Animals were tested either on the elevated plus maze and DRL (for males), or in IntelliCages (DDT then BST, 9 weeks in total).

### **3.3.4. Elevated plus maze**

The elevated plus maze consisted of two open (no wall) and two closed (with walls) arms (dark gray PVC, 30 cm length, 5 cm width) linked to a central platform elevated 60 cm above the ground. All experiments were carried out in red light (15W). Each mouse was placed on the central platform facing a closed arm, and allowed to explore the maze for 5 minutes. The latency to first enter an open arm, the time spent on each

arm and the total distance covered were automatically recorded by a videotracking system.

### 3.3.5. IntelliCage

Two-month old females were implanted with transponders (DATAMARS SA, Bedano/Lugano, Switzerland) under 5% isoflurane anesthesia to allow individual identification and monitoring in the IntelliCage. Following implantation, the animals were housed in standard home cages for one week then transferred to IntelliCages in groups of 16 per cage. The IntelliCage (NewBehavior AG, Zürich, Switzerland) is an automated testing device (20 cm height, 55 cm length, 38 cm width) with four conditioning corners. Each corner gives access to two bottles of liquid (one of water and one of 0.5% saccharine solution) through a hole (13 mm) (one bottle on each side of the hole). Each hole can be closed with an automated door to prevent access to bottles. The number and duration of nose-pokes are recorded by photobeams on both sides of each corner and drinking behavior is measured by lickometers. Access to water was limited according to protocols (see below), and food was available *ad libitum* in a food topper. Mice that did not adapt to the IntelliCage (no drinking for at least 36h during habituation) were removed from the cage and excluded from testing. Mice were habituated to an IntelliCage for 7 days during which they had free access to water in each corner. For training on the delay discounting task (DDT) (134), all doors were open and mice had free access to water and saccharine in each corner (0 second delay). DDT started when preference for saccharine was established (>50% saccharine licks over all licks). Upon every corner visit, doors giving access to water were always open but doors giving access to saccharine were open only after a delay (test phase). Doors opening was progressively delayed by an additional 0.5 seconds each day until the animals shifted their preference to water (<50% saccharine licks over all licks). At the end of training, doors giving access to water and saccharine were then re-open with no delay for 3 days to confirm the preference for saccharine. For the behavioral sequencing task (BST) (135), animals had access to drinking bottles for only 1h per day twice a day (11-12:00 and 16-17:00) for 7 days (doors were closed the rest of the time). For acquisition, each animal learned to alternatively visit two diagonal corners of the Intellicage to collect water (random assignment of diagonal corners for each animal). Re-entry to the same corner or visit to a corner of the other diagonal was not rewarded. Acquisition was followed by eight sessions of reversal for which corner assignment was switched to the other diagonal. Reversal was repeated 3 times. Body weight was checked weekly. The (i) number and duration of visits in

each corner (exploratory activity), (ii) number, duration and side (right or left) of nose-pokes, (iii) number, duration and side of licks, and duration and side of contacts between the mouse tongue and the bottle spout (considered as index of fluid consumption) were measured. In the DDT, preference for saccharine was evaluated by calculating a preference index  $(100*(S-W)/(S+W))$  with S=average lick contact time per saccharin choice, W=average lick contact time per water choice (100=consumption of saccharin only). For BST, the percentage of correct visits over total visits per test session was calculated to assess learning.

### **3.3.6. Delayed reinforcement of low rates (DRL) task**

One day before the experiment, animals had restricted water access. Four identical operant conditioning chambers (15,9 cm height, 16,5 cm length, 17,5 cm width) with a stainless steel grid floor (TSE Systems, Germany) were placed in sound-insulated boxes. Each chamber was equipped with a nose-poke response unit (2 cm diameter) fitted with a photocell sensor and a yellow cue light in the hole. A green cue light was located on the wall of the chamber above the nose-poke module. A regular light (2.8 W) located on the ceiling of the box was turned on to signal start and end of each testing session. A liquid dispenser fitted with a photocell sensor was located left to the nose-poke module. Each mouse was habituated to the chamber for 30 minutes, during which 10ul of water were delivered to the liquid dispenser every 30 seconds. The animals were then trained on a continuous reinforcement (CRF) schedule once daily, for which they had to nose-poke for the delivery of 10ul water during 30-minutes sessions until they reached a criterion of 50 nose-pokes per session. Following CRF, the animals were trained on a DRL phase. During this phase, nose-pokes were rewarded only if enough time (defined by a criterion: 6 or 18 seconds) had elapsed between two consecutive nose-pokes. The timer was reset after each reward delivery. Nose-pokes done too early (or too late) were not rewarded and reset the timer. A green cue light signaled when a nose-poke could have been rewarded. Each correct nose-poke turned on a yellow light as confirmation. The animals were first tested with a criterion time of 6 seconds for 3 consecutive weeks (15 testing days) then with a criterion of 18 seconds for 8 weeks (40 testing days). Testing was followed by three days of extinction during which the protocol was the same but the animals were not rewarded. The timing of each nose-poke was recorded by the photocell sensor (+/- 1 sec accuracy). Data analyses were conducted in Matlab. The distribution of inter-response time (IRT), which represents the interval between two successive nose-pokes, was calculated for each session. IRTs were normalized by dividing the number

of IRTs of > 1 seconds by the total number of IRTs. Extinction index is the difference in number of nose-pokes before extinction (last three days of training) and the third day of extinction. Body weight was checked weekly.

### **3.3.7. Active avoidance**

Active avoidance was tested using the same operant conditioning chambers as for DRL. Each chamber was equipped with a shock grid floor, and a nose-poke module fitted with a photocell sensor was placed on the left side of one of the chamber walls. A green cue light was located above the nose-poke module. A regular light (2.8 W) located on the ceiling of the box was turned on to signal start and end of each testing session. Mice were habituated to the chamber for 30 minutes during which the nose-poke module was closed. Two days after habituation, each mouse was trained to escape from a foot-shock (0.3 mA, max 10 seconds) by nose-poking over 60 trials for 30 minutes (one trial every 30 seconds). If the animal did not nose-poke, the foot-shock was automatically terminated after 10 seconds. During training, a normal light was always on, the green light was on when there was no shock, and the red light was on during shocks. The sensor located in the nose-poke hole recorded the time of occurrence of each nose-poke (1 seconds resolution). Latency to escape and number of escapes were measured then analyzed in blocks of 5 trials.

### **3.3.8. Drug administration**

The histone acetyl-transferase inhibitor C646(136) (4.45 $\mu$ g/kg) and the methyl-transferase inhibitor 3-deazaneplanocin A (3-DA)(67) (2mg/kg) in 0.9% saline were injected intraperitoneally (i.p.). Corticosterone (10 mg/kg, Sigma) in 3% ETOH (in 0.9% saline) and spironolactone (25 mg/kg, Sigma, previously reported to effectively block MR activity(137)) in 3% ETOH were injected i.p. daily for 7 days. Control mice received the same volume (10 ul/g) of 0.9% saline or 3% ETOH. 24 h after injection, the animals were behaviorally tested or sacrificed to extract the hippocampus for molecular analyses.

### **3.3.9. Quantitative RT-PCR**

DNasel-treated RNA (Allprep RNA/DNA kit; Qiagen) was reverse-transcribed (RT) using the SuperScript First-Strand Synthesis System II for RT-polymerase chain reaction (PCR; Invitrogen Carlsbad, California). Quantitative RT-PCR was performed

in an ABI 7500 thermal cycler using TaqMan probes targeting only exons common to the different splice variants (MR: Mm01241596\_m1\_m1, GR: Mm00433832\_m1, Applied Biosystems Foster City, California).

### **3.3.10. Western blotting**

Western blotting was performed as previously described (138). Briefly, 40 µg proteins from whole cell extracts were resolved on 4-12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked in milk 5% and then incubated in primary and secondary antibodies. GAPDH (1:1000; rabbit mAb; Cell Signaling) was used as internal control. Each primary antibody was run on a separate day. Membranes were then washed and incubated with secondary antibody goat anti-rabbit (IRDye 800nm, 1:10.000; Li-cor Biosciences). Band intensity was determined using an Odyssey IR scanner (Li-Cor Biosciences) and quantified with ImageJ. Samples from different groups were processed on the same blot. Data are expressed as percent relative to controls.

### **3.3.11. Bisulfite pyrosequencing**

Genomic DNA from hippocampus and sperm was extracted with Allprep kits (Qiagen) according to the manufacturer's instructions. Bisulfite pyrosequencing was performed by EpigenDx, Inc, USA. Universally methylated and unmethylated DNA samples (Millipore Bioscience Research Regents) were used as controls.

### **3.3.12. Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was performed as described previously(138) using anti-acetyl-histone H4 (Lys5) (H4K5ac, Millipore, ChIPAb), anti-acetyl-histone H3 (Lys14) (H3K14ac, Millipore, ChIPAb), anti-trimethyl-histone H3 (Lys36) (H3K36me3, Millipore), anti- dimethyl-histone H3 (Lys4) (H3K4me2, Millipore), anti-dimethyl-histone H3 (Lys9) (H3K9me2, Millipore), anti-dimethyl-histone H3 (Lys27) (H3K27me2, Millipore) or mouse IgG (Millipore, 12-371B). DNA was quantified in immunoprecipitated samples by quantitative PCR in an Applied Biosystems 7500 Thermal Cycler using specific primers (MR: forward, TGGGAAACTGGAAAAACCATC; reverse, CACTCTCACACATACACTCCTGCTG. GR: forward, GCCCCTCTGCTAGAGTGACA; reverse: GGAGGGAAAGCGAGTTCTT) and SYBR green Mastermix (QIAGEN) with

the following cycling conditions: 10 minutes at 94°C, 50 cycles of 20 seconds at 94°C + 1 minute at 60°C. Data were collected in the linear amplification range and each PCR experiment was repeated at least twice. Quantitative RT-PCR was performed for every immunoprecipitated sample, IgG and input control and samples were run in triplicates. GAPDH was used as control gene. The relative proportion of immunoprecipitated fragments was determined based on the threshold cycle value (Ct) for each PCR reaction. The following equation was used for normalization: Fold difference versus negative control =  $2^{-[(Ct\text{ Histone PTM}/Ct\text{ Input control}) - (Ct\text{ negative control}/Ct\text{ input control})]}$ .

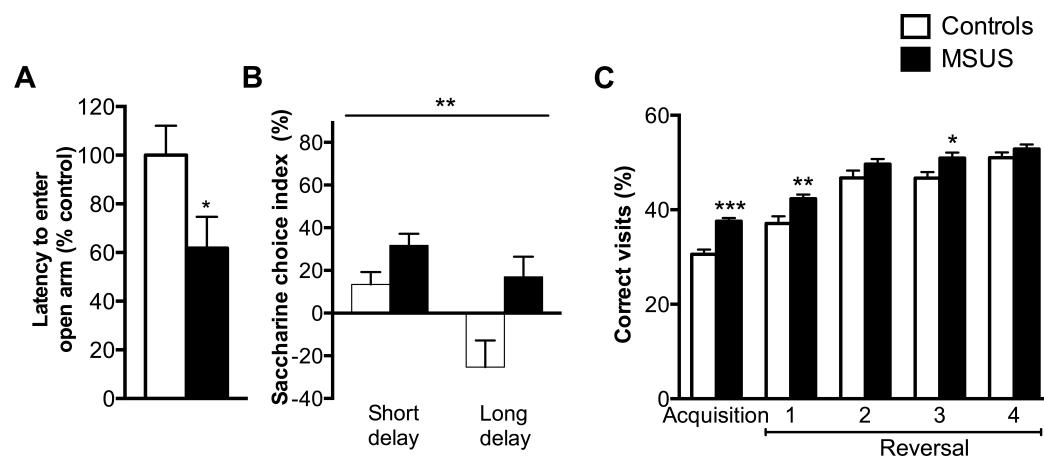
### **3.3.13. Statistical analyses**

Statistical analyses were performed using SPSS. Behavioral data from the elevated plus maze were analyzed using 2-tailed t-test. DRL and IntelliCages experiments were analyzed using repeated measurements ANOVAs with group (MSUS, control) as between subjects factor, and sessions or bins of sessions as within subject factors. Molecular experiments were analyzed by 2-tailed t-test. Outliers with a mean away from the group mean by +/- 2 standard deviation were excluded. Significance was set at 0,05.

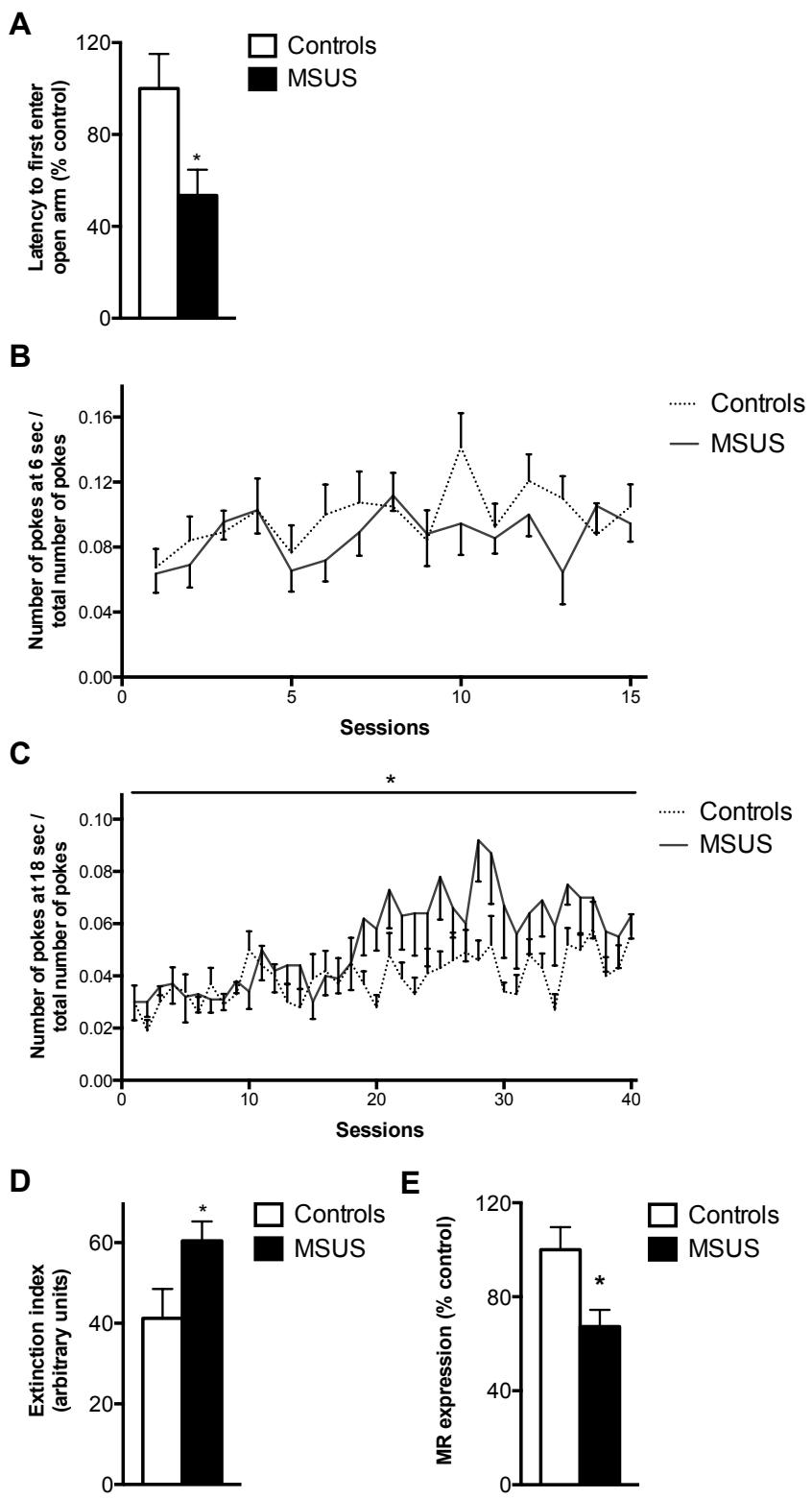
### 3.4. Results

We used a model of unpredictable maternal separation and maternal stress (MSUS) in the mouse (22) to examine the possibility that traumatic stress in postnatal life can have behavioral benefits in the offspring. Second-generation (F2) offspring were obtained by breeding males subjected to MSUS when pups (F1) to wild-type C57BL/6 adult females (Supplementary Fig. 1). The effect of MSUS on behavior in the F2 offspring was first examined using an elevated plus maze (EPM). This maze assesses approach/avoidance and response to risk and potential danger in aversive conditions (139). While control mice had a long latency to first enter an open arm, F2 MSUS animals had a short latency (Fig. 1A, Supplementary Table 1), indicating reduced aversion and avoidance. Similar behaviors were observed in F1 males (Fig. 2A), suggesting a comparable effect of MSUS in animals directly exposed to stress. Since such behavior may reflect changes in goal-directed or motivated behaviors, we next examined responses in non-aversive conditions using a delay-discounting task (DDT) in IntelliCages (140). This task assesses an animal's choice between a non-appealing but immediate reward (water) and an appealing but delayed reward (saccharine) based on a choice strategy. It also assesses impulsivity. While F2 control animals preferentially chose water and minimally delayed their choice for saccharine, F2 MSUS animals delayed their choice in favor of saccharine (Fig. 1B), suggesting improved goal-directed behaviors. This choice did not result from an increased preference for saccharine since MSUS animals had similar saccharine consumption to control mice in normal conditions (Supplementary Fig. 2). Because goal-directed behaviors depend on behavioral flexibility, in particular when rules change during a task (141), we next tested the animals on a behavioral sequencing task (BST), specifically designed to assess response flexibility, using IntelliCages. On this task, an animal is first trained to alternatively visit two opposite diagonal corners of an arena to obtain a drink reward. Corners assignment is then switched (reversal) to the other diagonal such that the animal has to shift shuttling from corners in one diagonal to corners in the other diagonal while maintaining a goal-directed strategy. On this test, F2 MSUS animals made more visits to the correct corners during both, the acquisition and the reversal phase (Fig. 1C), suggesting improved flexibility and better adaptation to new rules. A similar effect was observed in F1 males on a delayed reinforcement of low rates (DRL) paradigm (males cannot be tested on DDT or BST due to hierarchical fights resulting from group housing in IntelliCages that confound the results). On DRL, an animal can obtain a reward by nose poking in response to a cue after a predefined delay. With a 6-seconds delay, an easily attainable requirement (142), control and

MSUS mice nose-poked a similar number of times at the right delay, indicating proper response (Fig. 2B). However, with a 18-seconds delay, which is more demanding, F1 MSUS animals poked significantly more often than controls immediately after the cue onset (signaling that the required delay has elapsed), indicating improved goal-directed behaviors (Fig. 2C). Both groups however collected an overall similar number of rewards (data not shown). Further, during extinction, nose-pokes response was more rapidly extinguished in MSUS than control mice (Fig. 2D), suggesting a lower perseveration. In both F1 males and F2 females, the improved behavioral flexibility was not due to altered learning since response on an active avoidance test was similar in control and MSUS animals (Supplementary Fig. 3).



**Figure 1. Altered behavioral responses in F2 animals.** (A) Latency to first enter an open arm on the elevated plus maze (Controls n=16, MSUS n=15; t(29)=2.15). The results were replicated in two independent experiments. (B) Saccharine choice index in the DDT after a short (0,5-7 seconds) or long (7,5 seconds) delay (Controls n= 12, MSUS n=16; F(1,26)=8,21). (C) Percent of correct visits during acquisition and across the first 4 sessions of reversal on BST (Controls n=13, MSUS n=16; F(1,27)=8,05). Data are means  $\pm$  s.e.m. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. All experiments were performed in females because males cannot be tested on DDT and BST due to confounds by hierarchical fights.

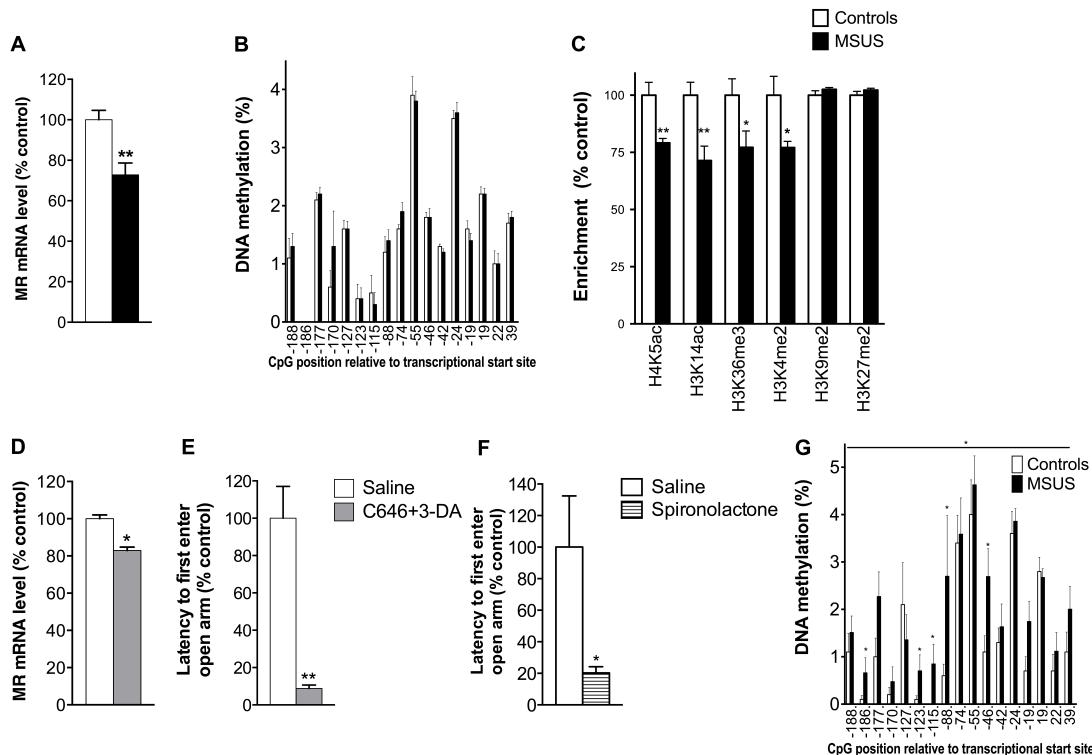


**Figure 2. Effect of MSUS on goal-directed behaviors and behavioral flexibility and MR expression F1 males.** (A) Latency to first enter an open arm on an elevated plus maze (Controls n=8, MSUS n=18;  $t(24)=0.79$ ). (B) Number of nose-pokes after the required delay (6 seconds) over the total number of pokes on the DRL task (Controls n=12, MSUS n=11;  $F(1,21)=1.05$ ) (C) Number of nose-pokes after the

required delay (18 seconds) over the total number of pokes on the DRL task (Controls n=11, MSUS n=11; F(1,20)=4.99). (D) Extinction index during extinction training on the DRL task (Controls n=9, MSUS n=9; t(16)=-2.19). (E) Quantitative RT-PCR showing MR expression in the hippocampus in F1 MSUS males (Controls n=7, MSUS n=8; t(13)=2.21) and females (Controls n=9, MSUS n=7; t(14)=2.57). \*p<0,05.

Previous studies have implicated components of the hypothalamic-pituitary-adrenal (HPA) axis and their epigenetic regulation in the effects of early stress on behavior (11, 22). We examined whether the MR, recently implicated in goal-directed behaviors (143) and in the appraisal of novel environments (144), is associated with the effects of MSUS. Quantitative RT-PCR and Western blot showed that MR mRNA (Fig. 3A) and protein (Supplementary Fig. 4) respectively were significantly lower in the hippocampus of F2 MSUS mice, a brain structure involved in adaptive learning and behavioral control (145), compared to controls. This decrease was also observed in F1 fathers (Fig. 2E) (and F1 female siblings (Supplementary Fig. 5)), suggesting a consistent down-regulation across generations and sex. The olfactory bulb, a brain region unrelated to behavioral flexibility did not show any down regulation of MR (Supplementary Fig.6). Since gene regulation induced by environmental factors implicates epigenetic processes, we next examined DNA methylation at the MR promoter. Quantitative bisulfite pyrosequencing showed that DNA methylation upstream of the transcription start site and important for transcriptional regulation (146) was similar in male F1 and female F2 control and MSUS hippocampus (Supplementary Fig. 8B and Fig. 3B). We then examined histone posttranslational modifications (HPTMs), other important epigenetic marks influenced by stress (11, 66) and associated with transcriptional regulation in the adult mouse hippocampus (138). Chromatin immunoprecipitation (ChIP) assays showed that acetylation of H4K5 (H4K5ac) and H3K14 (H3K14ac), dimethylation of H3K4 (H3K4me2), and trimethylation of H3K36 (H3K36me3) were significantly decreased at the MR gene in F2 MSUS hippocampus, while H3K9 (H3K9me2) and H3K27 (H2K27me2) dimethylation was not altered (Fig. 3C). The decrease was specific to MR and was not observed at the GR gene (Supplementary Fig. 7A), consistent with unaltered expression of this gene (Supplementary Fig. 7B). No change in HPTMs was observed at the MR gene in F1 males (Supplementary Fig. 8A). We confirmed that altered HPTMs in F2 hippocampus are relevant for MR expression by mimicking their alteration pharmacologically *in vivo*. Injection of inhibitors of histone acetyl- and methyl-transferases (C646 and 3-deazaneplanocin A, 3-DA), known to block histone

acetylation and methylation in the brain (147), in adult animals decreased MR expression in the hippocampus (Fig. 3D) without affecting GR expression (Supplementary Fig. 7C). MR regulation was also functionally relevant for behavior since animals treated with the inhibitors had a decreased latency to first enter an open arm on the EPM, similarly to MSUS mice (Fig. 3E, Supplementary Table 1). Consistently, blockade of the MR receptor by chronic injection of an MR competitive antagonist spironolactone similarly decreased the latency to first enter an open arm on the EPM (Fig. 3F, Supplementary Table 1). Real-time quantitative RT-PCR also showed that spironolactone significantly decreased MR expression but did not affect GR expression (Supplementary Fig. 10A), consistent with the ability of MR to regulate its own expression (148). The effect of the drugs on behavior was not due to an increased activity since the total distance covered on the maze was not altered by the inhibitors (Supplementary Fig. 9) or by spironolactone (Supplementary Fig. 10B). These results therefore strongly suggest a relationship between MR expression and behavioral alterations induced by MSUS. Finally, since DNA methylation in germ cells has been implicated in epigenetic inheritance (22, 48, 149), we examined its profile at the MR promoter in the sperm of F1 animals. Bisulfite pyrosequencing showed that DNA methylation was increased at several CpGs across the MR promoter (Fig. 3G), suggesting a potential implication in transmission.



**Figure 3. Epigenetic regulation of MR expression.** Epigenetic regulation of MR expression. (A) Level of MR mRNA in the hippocampus of F2 animals measured by

quantitative RT-PCR (Controls=12, MSUS n=7; t(17)=3.54). These results were reproduced in two independent experiments. (B) Percent methylated cytosines for each CpG at the MR promoter in the hippocampus of F2 animals (Controls n=6, MSUS n=7; F(1,11)=0,56). Numbers below the x-axis indicate CpG site position. (C) ChIP assays showing H4K5ac (Controls n=6, MSUS n=6; t(10)=6.21), H3K14ac (Controls n=6, MSUS n=6; t(10)=3.36), H3K36me3 (Controls n=5, MSUS n=6; t(8)=2.25), H3K4me2 (Controls n=6, MSUS n=5, t(9)=2.43), H3K9me2 (Controls n=4, MSUS n=4, t(6)=-1.2) and H3K27me2 (Controls n=4, MSUS n=4, t(6)=-1.198) in the hippocampus of F2 animals. (D) MR mRNA level after injection of the histone acetyl- and methyl-transferases inhibitors, C646 and 3-DA, compared to saline (Saline n=4, C646+3-DA n=5; t(4.85)=6.47). (E) Latency to first enter an open arm on the elevated plus maze in animals treated with C646 and 3-DA (Saline n=8, C646+3-DA n=9; t(23,6)=5,31). (F) Latency to first enter an open arm on the elevated plus maze in animals treated with saline or spironolactone (Saline n=8, Spironolactone n=9; t(7,21)=2,44). (G) Percent methylated cytosines for each CpG at the MR promoter in the sperm of F1 males (Controls n=11, MSUS n=6; F(1,15)=6,77). Numbers below the x-axis indicate CpG site position. Data are means  $\pm$  s.e.m. \*p $\leq$ 0.05, \*\*p $<$ 0.01. Most molecular experiments were carried out in females for consistency with behavior.

### 3.5. Discussion

Many paradigms of early life stress have focused on acquired maladaptive traits (5). For instance early life stress caused by reduced nesting material leads to increased stress reactivity and impaired learning and memory on the Morris water maze task (150). It is now however accepted that the programming effect of early life environment, critically depends on the environment encountered later on (64) and that the consequences of chronic stress exposure can not be generally categorized as positive or negative. They have to be interpreted for each analyzed trait separately (151). In this line, in the current study we propose a nuanced view on the impact of MSUS on behavior. Previous publications on our model of early life stress reported on negative consequences, such as depressive like behaviors, in response to MSUS (22, 152, 153). Here we suggest that traumatic stress experienced in early life can additionally confer some adaptive advantage to the progeny and improve behavioral flexibility in challenging situations. Another form of learning in an active avoidance paradigm was found to be unaffected in F1 males and F2 females, again highlighting the importance of a very detailed and refined interpretation.

The mismatch model of stress reactivity proposes that individuals will cope best with an environment that they had been exposed to already earlier in life (64). Evolutionary

interpretations of the mismatch model propose that early life environment induces persistent changes with adaptive value (79). Early life stress could thus help an individual cope with stressful events later in life. Our results significantly extend the mismatch model since they introduce a heritable component to the model and show that the offspring can also benefit.

The data further provide evidence for the involvement of epigenetic regulation, since the pharmacological mimic of altered histone PTMs copies the altered behavior on the EPM observed in F2 MSUS animals. Further, the fact that histone PTMs are altered in the hippocampus of F2 MSUS animals at the MR gene, suggests that MR is an HPA axis component subjected to such regulation. This interpretation is further strengthened by the finding, that the same pharmacological intervention of histone PTMs, which mimicked the behavioral phenotype, simultaneously decreased MR expression. These findings further point to a role for MR in behavioral adaptation and in its persistence across generations. It is highly unlikely that MR is the only gene that mediates the effect of MSUS on behavioral flexibility, as alterations in complex behaviors are known to involve a wide range of genes (154). Yet, since pharmacological blockage of the MR receptor mimicked the increased approach behavior, we can conclude that the MR receptor is crucially involved to the altered behavior induced by MSUS.

Our study suggests that DNA methylation in germ cells may be implicated in the transmission of altered MR expression, since we demonstrate altered DNA methylation at the MR promoter in sperm cells. This corroborates previous findings in this model that showed an association between altered gene expression of the cannabinoid receptor, the corticotrophin releasing factor receptor 2, and methyl CpG binding protein2 in the cortex of F2 MSUS animals with altered DNA methylation at the promoter of these genes in the same brain region of these animals and in the germline (22). However, the DNA methylation is not altered at the MR gene in the hippocampus of F2 MSUS females, but HPTMs are. Thus altered DNA methylation in the germ cell might constitute an intermediate, which is later on converted into another epigenetic mark. Additionally, sperm HPTMs may constitute another means of transmission. Most of histones get replaced in sperm by protamines, yet a small proportion resists the replacement (155). Interestingly H3K4me2 can be retained at the MR promoter in spermatozoa (156), suggesting a transmission of altered MR expression via retained HPTMs at the MR gene in the male germ line.

Finally, the findings are highly relevant to the clinic since MR is linked to stress-induced pathologies like depression (157, 158) in human. They may open novel perspectives for potential therapeutic treatment in the future.

### **3.6. Acknowledgments**

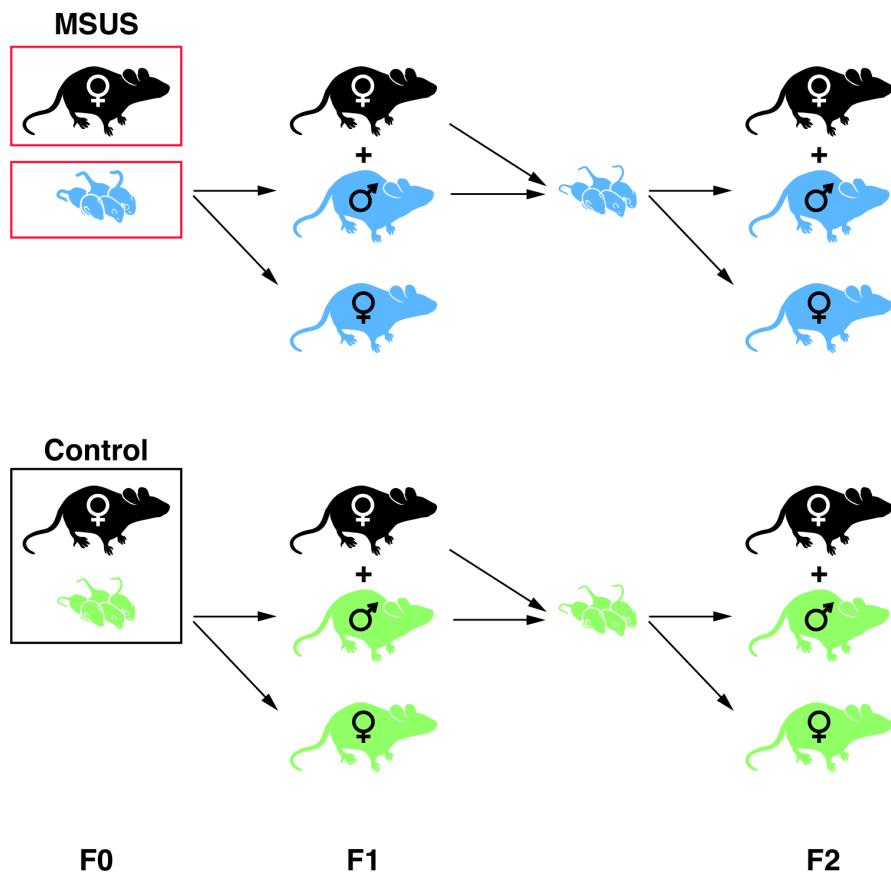
This work was supported by the Austrian Academy of Sciences, the University Zürich, the Swiss Federal Institute of Technology, Roche, the Swiss National Science Foundation, and The National Competence Center for Research “Neural plasticity and Repair”. J.Bohacek is supported by a postdoc ETHZ fellowship and a Roche fellowship. K. Gapp is supported by a DOC-fFORTE Fellowship from the Austrian Academy of Science. We thank Elisabetta Vanoni for instructions with IntelliCages, Heiko Hörster for help with animals, Safa Mohanna for Matlab data analyses, Melly Oitzl for help with the interpretation of behavioral experiments and Hans Welzl for helpful comments on the manuscript.

### **3.7. Authors' contribution**

K.G. performed mRNA and DNA experiments. K.G. and M.A. performed IntelliCages experiments, and K.G. and T.F., EPM experiments. S.S. and H.S. performed chromatin immunoprecipitation. G.V. performed spironolactone injections and helped with mRNA experiments. D.P.W. did statistical analyses of DDT data. J.B. prepared the animals. K.G. and I.M.M. designed the experiments, interpreted the results and wrote the manuscript.

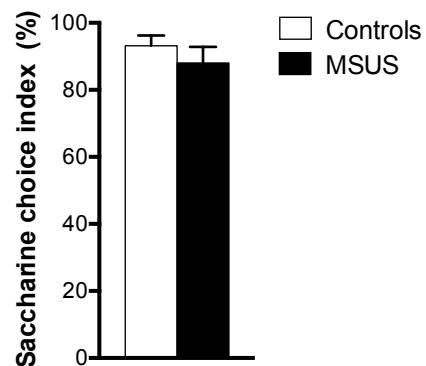
### 3.8. Supplementary Material

Supplementary Figure 1 (Gapp et al.)



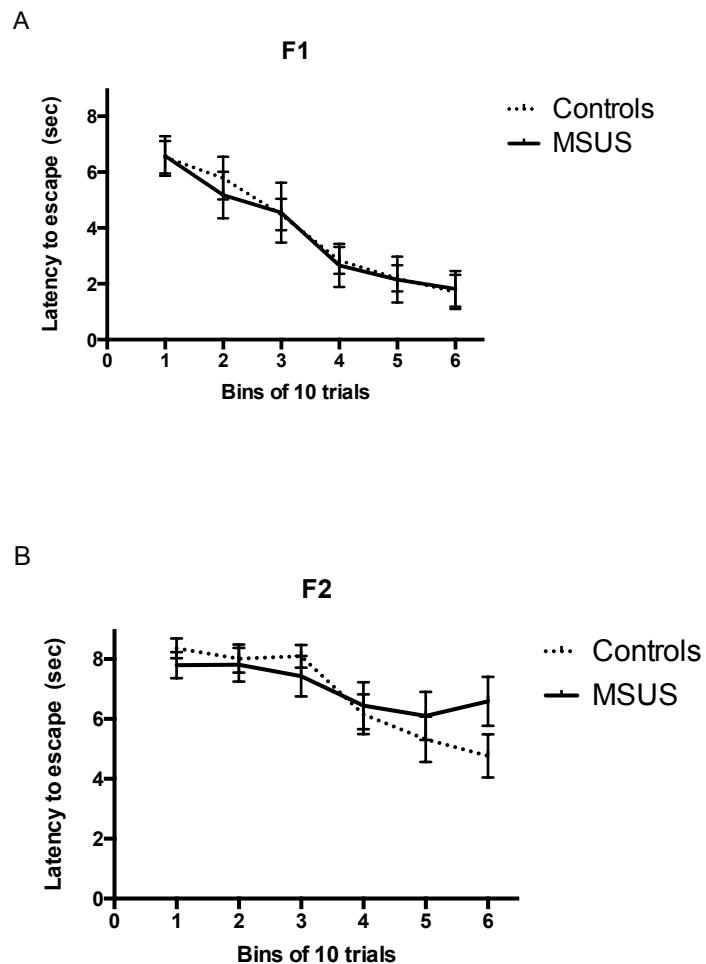
**Experimental design.** C57BL/6 F0 females (left boxes) bred to C57BL/6 males are subjected to MSUS or allowed to raise their offspring in regular conditions (bottom) from postnatal day (PND) 1 to 14 (top). Males from F1 offspring are then bred with naïve C57BL/6 females to obtain a second generation (F2) that is raised in normal conditions (no maternal separation or maternal stress). Behavioral testing is done in F1 males and F2 females when adult.

**Supplementary Figure 2 (Gapp et al.)**



**Saccharine preference in F2 females.** Saccharine choice index in the DDT during free access to water and saccharine . 0 reflects no preference for saccharine and values >0 reflect natural preference for saccharine (Controls n= 12, MSUS n=16;  $t(12.15)=1.19$ ).

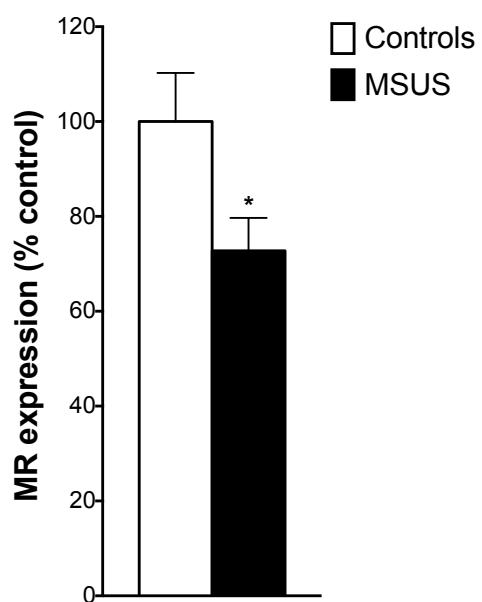
**Supplementary Figure 3 (Gapp et al.)**



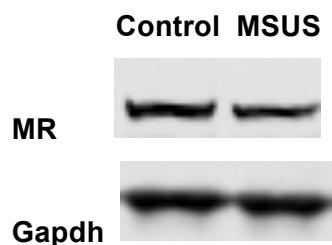
**Active avoidance performance.** Latency to escape from a foot-shock by nose poking in (A) F1 males (Controls n=11, MSUS n=11;  $F(1,20)=0.02$ ) and (B) F2 females (Controls n=16, MSUS n=16;  $F(1,30)=0.24$ ) across 6 blocks of 10 trials.

**Supplementary Figure 4 (Gapp et al.)**

**A**

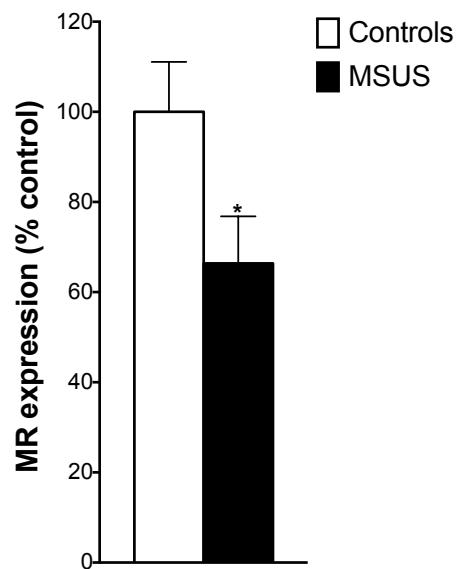


**B**



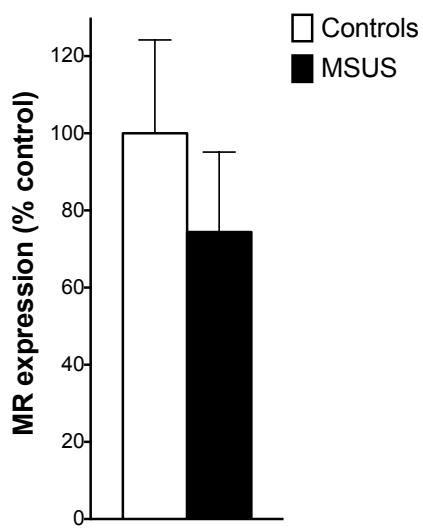
**MR protein expression in F2 females.** Western blot showing MR protein expression in the hippocampus in F2 MSUS females (Controls n=7, MSUS n=7;  $t(2,2)=12$ ). \* $p<0,05$ .

**Supplementary Figure 5 (Gapp et al.)**



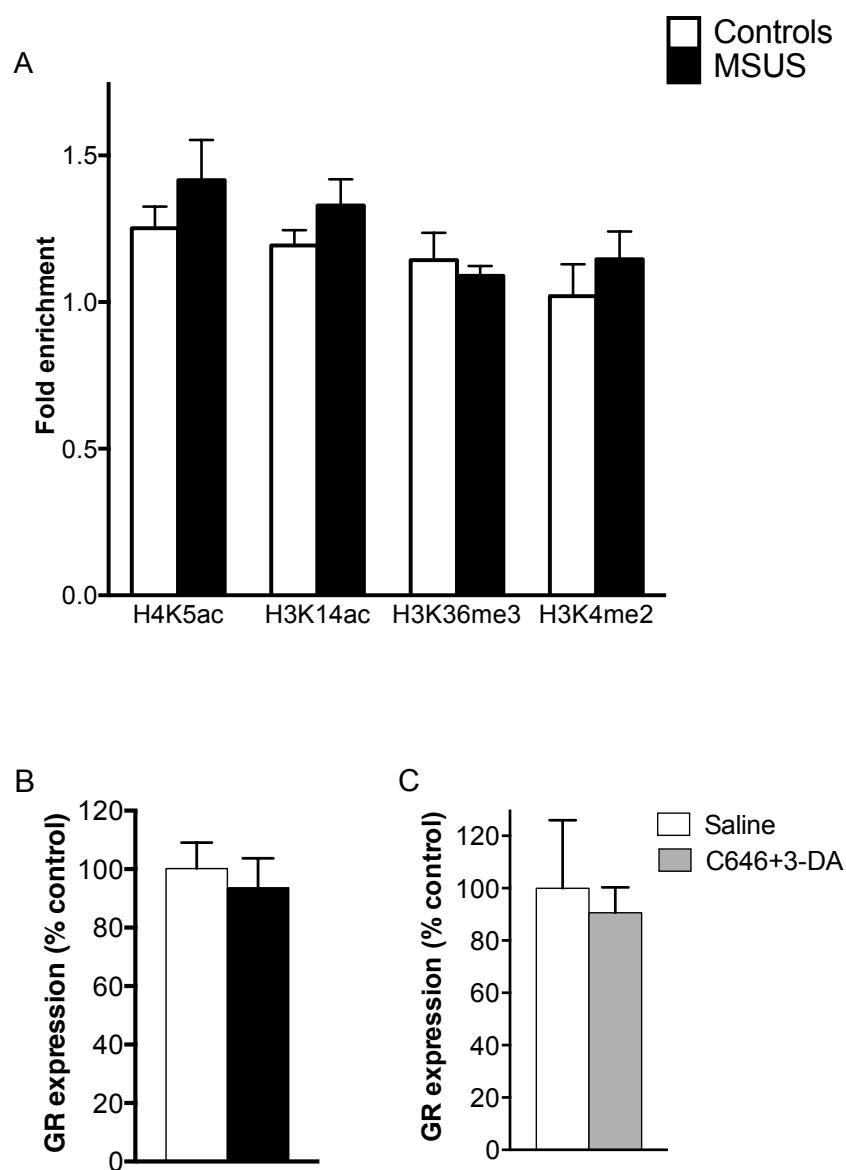
**MR expression in F1 females.** Quantitative RT-PCR showing MR expression in the hippocampus in F1 MSUS females (Controls n=9, MSUS n=7;  $t(14)=2.57$ ). \* $p<0,05$ .

**Supplementary Figure 6 (Gapp et al.)**



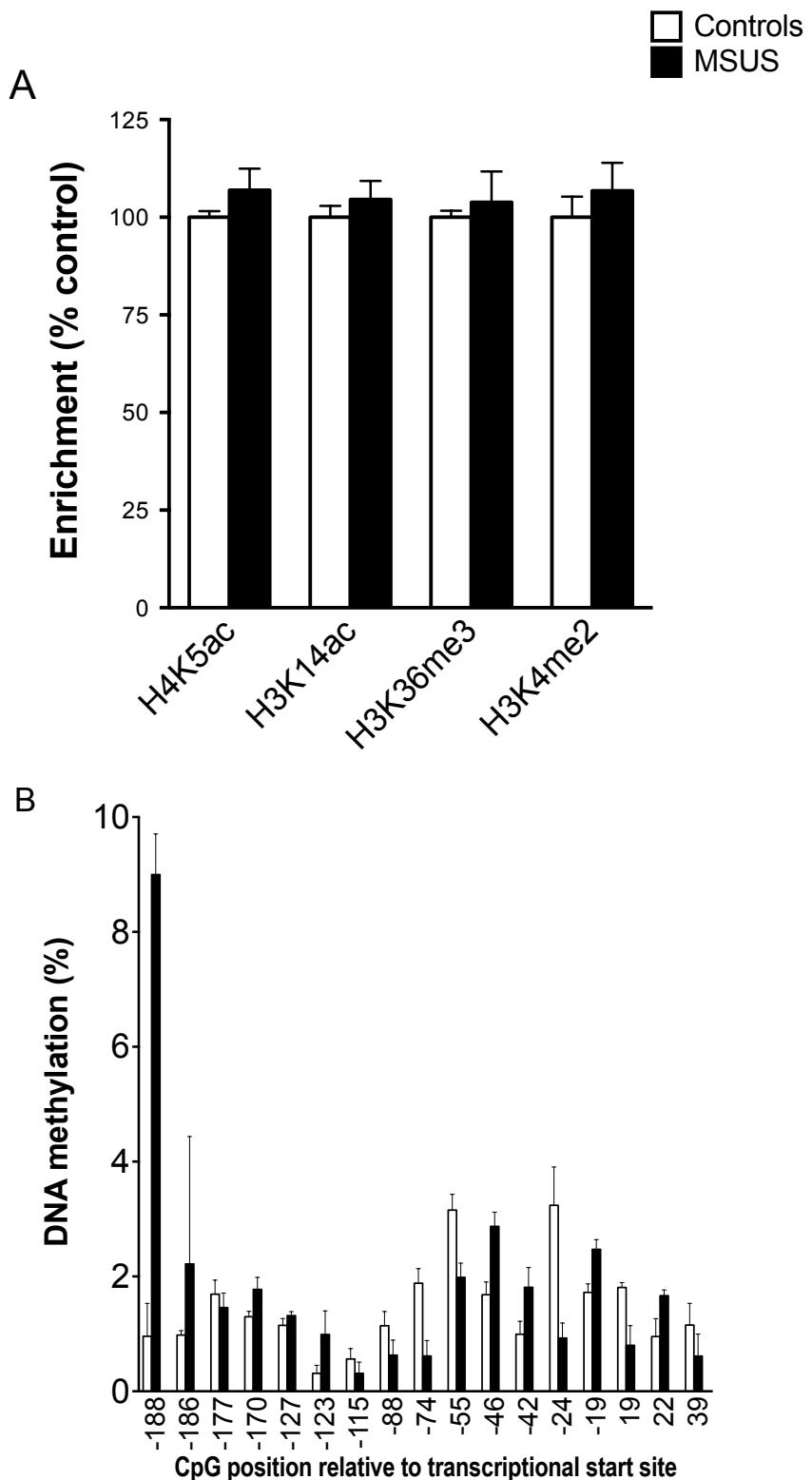
**MR expression in F2 female olfactory bulb.** Quantitative RT-PCR showing MR expression in F1 MSUS females (Controls n=7 MSUS n=8;  $t(13)=0.67$ ;  $p=0.43$ ).

**Supplementary Figure 7 (Gapp et al.)**



**Epigenetic and expression analyses of GR gene.** (A) ChIP assays showing histone PTMs at the GR gene in the hippocampus in F2 females (H4K5ac (Controls n=6, MSUS n=6; t(6)=-1.05), H3K14ac (Controls n=6, MSUS n=6; t(6)=-1.33), H3K36me3 (Controls n=5, MSUS n=6; t(3)=0.66), H3K4me2 (Controls n=6, MSUS n=5; t(1)=-0.61), H3K9me2 (Controls n=4, MSUS n=4; t(6)=-.863) and H3K27me2 (Controls n=4, MSUS n=4; t(6)=0.51)). (B, C) Quantitative RT-PCR showing GR expression in the hippocampus of (B) F2 MSUS females (Controls n=7, MSUS n=6; t(11)=0.48) and (C) F2 MSUS females after injection of inhibitors of histone acetyl- and methyl-transferases compared to saline (Saline n=4, C646+3-DA n=5; t(6)=0.25). Data are means  $\pm$  s.e.m.

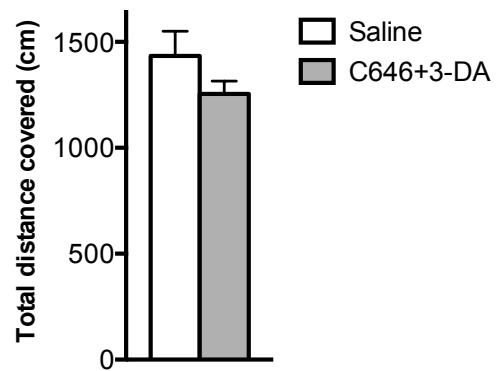
**Supplementary Figure 8 (Gapp et al.)**



**Histone PTMs and DNA methylation at MR gene in the hippocampus of F1 males.** (A) ChIP assays showing HPTMs at the MR gene in the hippocampus (H4K5ac (Controls n=6, MSUS n=8; t(6)=-1.05), H3K14ac

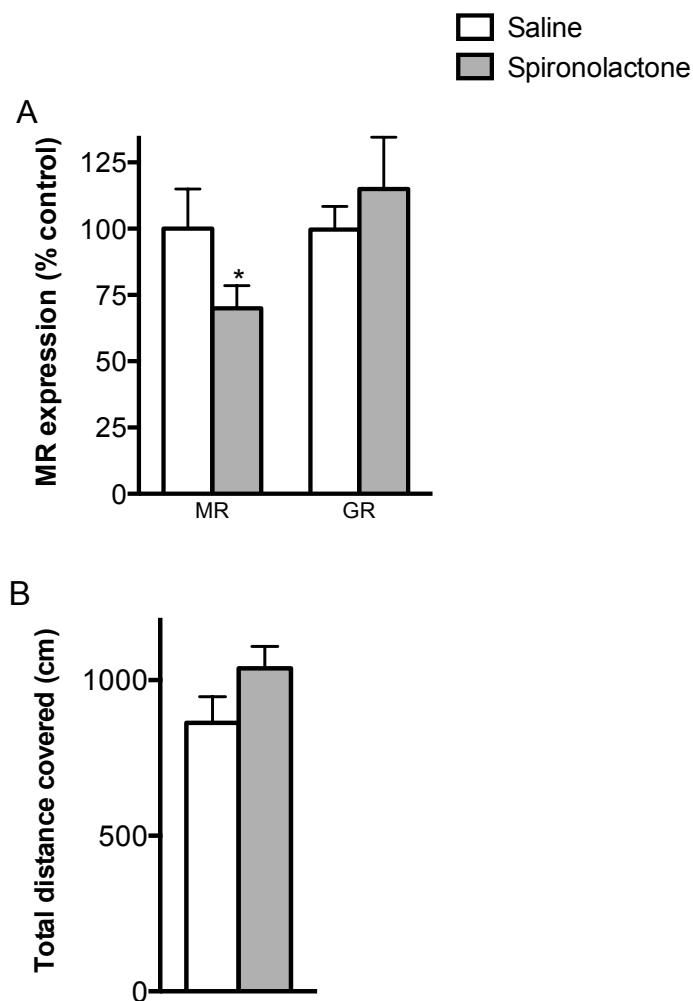
(Controls n=6, MSUS n=8; t(6)=-1.33), H3K36me3 (Controls n=5, MSUS n=8; t(3)=0.66), H3K4me2 (Controls n=6, MSUS n=8; t(1)=-0.61) (B) Percent of methylated cytosines for each CpG at the MR promoter in the hippocampus (control n=6, MSUS n=5; F(1,9)=0,1). Data are means ± s.e.m.

**Supplementary Figure 9 (Gapp et al.)**



**Activity on an elevated plus maze.** Total distance covered on an elevated plus maze in adult females injected with saline ( $n=26$ ) or with inhibitors of histone acetyl- and methyl-transferases C646+3-DA ( $n=10$ ) ( $t(33,51)=1,36$ ). Data are means  $\pm$  s.e.m..

**Supplementary Figure 10**



**Effect of spironolactone injection on (A) expression of MR ( $t(21)=2.03$ ) and GR ( $t(21)=0.85$ ) in animals injected with saline ( $n=8$ ) or spironolactone ( $n=9$ ), and (B) activity on an elevated plus maze assessed by total distance covered in adult mice injected with saline ( $n=10$ ) or spironolactone ( $n=10$ ) daily for 7 days ( $t(18)=-1.6$ ) and Data are means  $\pm$  s.e.m.**

\* $p=0.05$ .

**Supplementary Table 1 (Gapp et al.)**

Elevated plus maze		Latency to first enter an open arm					
		Controls		MSUS			
		Mean	Standard error of the mean	Mean	Standard error of the mean		
F1	Males	230.4	34.8	123.2	25.8	$t(24)=0.79, p<0.05$	
F2	Females	231.0	28.1	142.4	29.8	$t(29)=2.15, p<0.05$	
		Saline		Drug			
		Mean	Standard error of the mean	Mean	Standard error of the mean		
		C646+3-DA drugs injection	Females	156.7	26.7	13.9	2.8
Spironolactone injection	Females	134.1	43.4	27.2	5.3	$t(7,21)=2.44, p<0.05$	

**Performance of F1 and F2 animals on an elevated plus maze.** Mean raw values in seconds and corresponding standard error for latency to first enter an open arm on the elevated plus maze in F1 MSUS males, F2 MSUS females and respective controls injected with the histone acetyl- and methyl-transferases inhibitors (C646+3-DA), or with spironolactone compared to saline.

## **4. Environmental enrichment counteracts the trans-generational transmission of the effects of early life stress on mood and anxiety-related behavior**

Katharina Gapp<sup>1</sup>, Johannes Bohacek<sup>1</sup>, and Isabelle M. Mansuy<sup>1\*</sup>

<sup>1</sup> Brain Research Institute, University of Zürich and Swiss Federal Institute of Technology

\* Corresponding author, Email: mansuy@hifo.uzh.ch

*Keywords (7): maternal separation, environmental enrichment, inheritance of acquired traits*

**To be submitted at Neuroscience**

## **4.1. Abstract**

Traumatic early life stress constitutes a risk factor for the development of neuropsychiatric disorders later in life and across generations. In mice, early life stress has been shown to induce depressive-like behaviors in the exposed animals and in their offspring. In contrast, stimulating environmental conditions have been reported to positively impact behavior and may ameliorate the negative consequences of aversive experiences. Here we assess whether the exposure of male mice to early life stress causes behavioral alterations in their offspring, and whether environmental enrichment following early life stress can reverse these effects on the offspring. Mice were exposed to unpredictable maternal separation and maternal stress for the first two weeks of life, or raised under control conditions. Subsequently, half of the mice in each group were raised in enriched environmental conditions or under standard housing conditions. When adult, the males were tested on the elevated plus maze and subsequently bred to naïve females to generate offspring. Offspring were raised without further experimental manipulations until adulthood and then tested on the elevated plus maze, open field test and forced swim test.

We find that both unpredictable maternal separation stress and environmental enrichment have trans-generational effects on anxiety and depression-like behavior in mice. Further, environmental enrichment reverses the depressive-like behaviors induced by early life stress. These findings emphasize the positive impact of stimulating environmental conditions on behavior and demonstrate their therapeutic potential to prevent the inheritance of predisposing risk factors for mental illness.

## **4.2. Introduction**

Environmental factors profoundly influence the development and expression of behavioral traits in many species, including rodents and humans. Traumatically stressful experiences early in life strongly increase the risk to develop neuropsychiatric disorders later in life, including depression, anxiety disorders and cognitive deficits (159, 160). Epidemiological studies underscore the impact of aversive experiences on the onset of maladaptive behaviors in the offspring, even if they themselves were not exposed to trauma (38, 74, 161, 162). We have previously demonstrated non-genomic transmission of the impact of traumatic early life stress across generations in mice (22, 152, 153), and similar effects have since been described by others (163, 164). Among the complex effects of childhood trauma on the exposed mice as well as on their offspring, early life stress can alter the response to stressful and aversive situations and induce depressive-like behaviors (127, 165).

In contrast to the detrimental effects of early life stress, stimulating environmental conditions in early life can have beneficial effects on emotional and cognitive behaviors (87, 166). Recent studies have suggested that some of the beneficial effects of environmental enrichment (EE) can also be transmitted to the offspring (35), and that this may counteract the negative effects induced by early life stress (36). However, as these studies have been conducted in females, it is not clear whether the transmission to the offspring occurs through effects on the germ line or whether alterations in maternal care or in-utero fetal programming account for the transmission (127, 167). Thus, we tested whether effects of environmental enrichment (EE) can also be transmitted through the male line and whether it can counteract the negative impact of an unpredictable maternal separation and maternal stress paradigm (MSUS) on behavior of the subsequent generation.

We find that MSUS alters anxiety levels in the exposed animals, and in their offspring alters risk assessment and anxiety-related behaviors and induces depressive-like behavior. Additionally, we show that EE reduces anxiety behaviors in the exposed animals, and increases locomotor activity in their offspring. Most importantly, we find that environmental enrichment can counteract the detrimental effects of early-life stress on the offspring, as it reverses depressive-like behaviors induced by unpredictable maternal separation.

### **4.3. Methods**

#### **4.3.1. Animals**

C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature- and humidity-controlled facility. Cages housing 3-5 mice were cleaned weekly, and water and food were provided ad libitum. Animals were randomly assigned to standard housing or environmentally enriched housing conditions upon weaning. All experimental manipulations were performed during the animals' active cycle and in accordance with guidelines and regulations of the cantonal veterinary office, Zurich.

#### **4.3.2. MSUS treatment and breeding**

For unpredictable maternal separation with maternal stress (MSUS), first generation (F1) pups were subjected to daily 3-hr proximal maternal separation from PND1 to PND14. During separation, mothers and pups were placed in separate clean cages with bedding. Litters and dams were placed such that they had visual and olfactory contact. The timing of separation was unpredictable, but always occurred during the

dark cycle. Maternal stress consisted of either 20-min restraint in a Plexiglas tube or 5-min forced swim in cold water (18°C) applied unpredictably and randomly during separation. Both MSUS and control dams and litters had their cages changed on PND1, PND7, PND14, and PND21, during which the pups were also weighed. Apart from this manipulation, control dams and litters were left undisturbed. Only dams giving birth less than a week apart of each other were used. Litters with less than 4 pups were excluded from the experiment. When adult, males subjected to MSUS and controls (F1) were bred to naïve wild-type females to produce an F2 generation. F2 mice were reared in normal conditions not involving any stress.

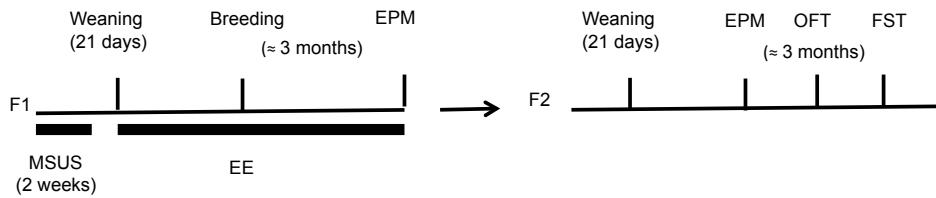
#### **4.3.3. Housing conditions**

MSUS and control mice were always housed in separate cages. Control and MSUS mice in standard housing (SH) were housed in groups of 3-4 animals per cage in Nalgene cages (32x17x14 cm) containing wood chip bedding and a small carton house. Control and MSUS mice (males) subjected to environmental enrichment (EE) were placed in social groups (n=12) in an enriched cage immediately after weaning until adulthood (Marlau©, Viewpoint). Behavioral testing was carried out after EE. The enriched cage consisted in a large box with two levels (55x36x19cm lower and 55x36x11cm upper floor). The bottom level was separated into two compartments, one containing food pellets, and the other with access to water and containing running wheels, and a covered/protected area. The top level consisted of a maze (35x36x11cm) whose shape and configuration were changed three times per week with a total of 12 different options. The box was organized such that animals had to go through the maze to reach the food compartment.

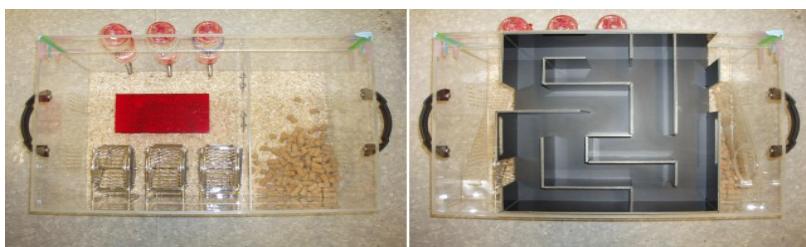
#### **4.3.4. Behavioral Testing**

Throughout all behavioral testing the experimenter was blind to treatment and tracking was performed manually as well as automatically (Viewpoint System). Mice were tested under indirect, dim red light when animals were 3 months old. F1 generation mice were tested on the EPM after breeding. F2 mice were tested in the following order on the elevated plus maze test, the open field test, and forced swim test. The timeline of the experimental design is summarized and outlined in Figure 1.

A



B



**Figure 1.** (a) Timeline representing manipulations (MSUS and EE), behavioral testing and breeding. (b) Top-down view of cages used for EE (Viewpoint, France) showing bottom (left) and top level (right). EPM elevated plus maze, OFT open field test, FST

#### 4.3.5. Elevated Plus Maze (EPM)

The EPM consisted of two open arms (without walls) and two closed arms (with walls), elevated 60 cm from the ground. Light conditions were kept at 15W red light throughout testing. Each mouse was placed on the central platform, facing a closed arm, and observed for a 5-min period. The latency to enter open arms, the time spent in each type of arm and the total distance moved were automatically recorded. The incidence of rearing, protected and unprotected stretch-attend postures at the center of the maze were manually recorded. After each trial, the EPM apparatus was wiped with tissue to remove feces and to disperse olfactory cues.

#### 4.3.6. Open field test (OFT)

Mice were placed in an open field (novel, 71 x 71.5 x 31 cm; repeated, 64 x 51 x 46.5 cm) and allowed free exploration for 10 min or repeatedly across 2 days, for 5 min, 3 times. Latency to enter the center of the open field, and distance covered were quantified using automated scoring system (Viewpoint).

#### **4.3.7. Forced swim test (FST)**

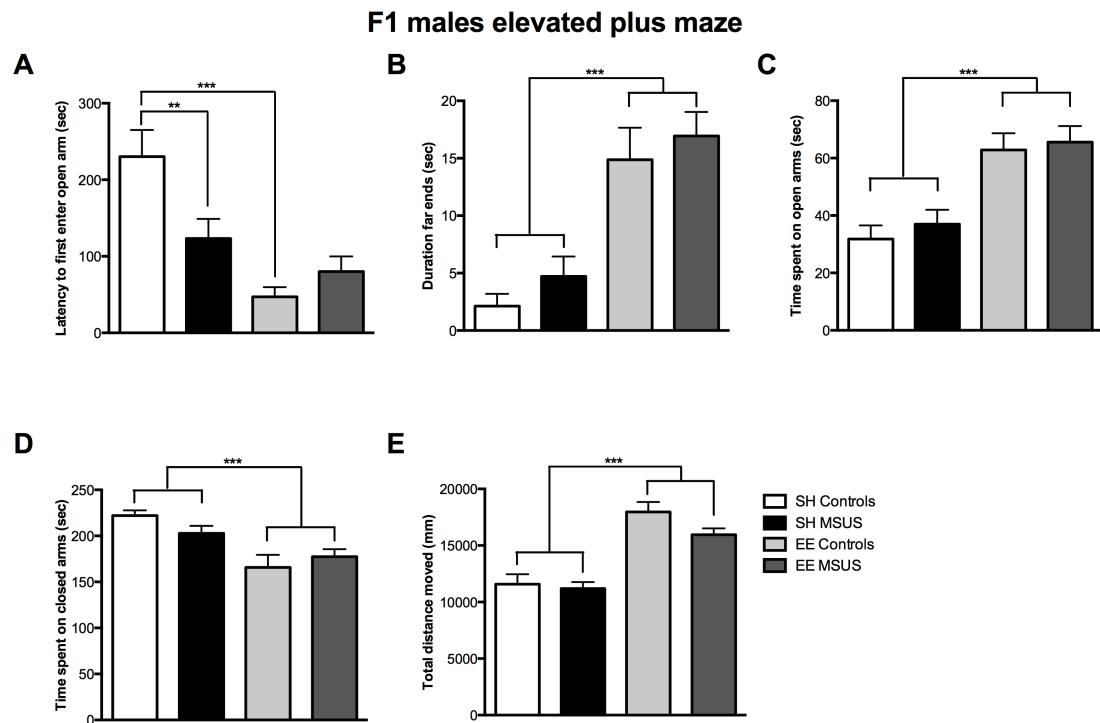
Mice were placed in a small tank of water (24 cm high, 19 cm diameter,  $18\pm1^{\circ}\text{C}$ , filled up to 18 cm) for 6 min. Manual scoring was done to determine the time the animal spent floating during the last 4 min of the test.

#### **4.3.8. Statistical analyses**

The study design resulted in the following four groups and group sizes in the parent generation (F1): 1) standard-housed control mice (SH-controls, n=8), 2) standard-housed MSUS mice (SH-MSUS, n=18), 3) environmentally enriched control mice (EE-controls, n=8), 4) environmentally enriched MSUS mice (EE-MSUS, n=11). Breeding resulted in the following group numbers in the offspring (F2 generation): SH-controls (n=20), SH-MSUS (n=20), EE-controls (n=20), EE-MSUS (n=16). All behavior tests were analyzed with two-way ANOVAs with main factors of stress (control and MSUS) and housing (SH and EE). Fisher's LSD post-hoc tests were used when appropriate. Significance was set at  $p<0.05$  for all tests. Error bars represent standard error of the mean (SEM).

## 4.4. Results

### 4.4.1. F1 generation



**Figure 2:** The effect of EE on anxiety-like behaviors in mice exposed to MSUS. (a) latency to first enter an open arm (b) time spent on extreme ends of open arms (c) time spent on open arms (d) time spend on closed arms (e) total distance moved on the EPM.

### EPM

Increased anxiety is a trait observed in many neuropsychiatric disorders and has previously been shown to be affected by MSUS (153). Thus, to confirm treatment efficacy, we assessed anxiety behaviors using the elevated plus maze (EPM) in adult F1 males before breeding.

Two-way ANOVA showed a significant main effect of housing on the latency to enter the open arms ( $F(1,37) = 15.968$ ,  $p < .001$ ) (Fig. 2a), on the duration on the far ends of open arms ( $F(1,37) = 45.349$ ,  $p < .001$ ) (Fig. 2b), on the time spend on the open arms ( $F(1,37) = 35.68$ ,  $p < .001$ ) (Fig. 2c), on the time spent on the closed arms ( $F(1,37) = 25.755$ ,  $p < .001$ ) (Fig. 2c) and on the total distance moved ( $F(1,37) = 55.043$ ,  $p < .001$ ) (Fig. 2e). There was a significant interaction between stress x housing for the latency to enter the open arms ( $F(1,37) = 5.785$ ,  $p = 0.021$ ). Follow-up post-hoc tests revealed that EE-controls had a lower latency to enter the open arms than SH-controls ( $p <$

0.001), but there was no difference between SH-MSUS and EE-MSUS mice ( $p = 0.211$ ). Further, SH-MSUS mice had a lower latency to enter the open arms when compared to SH-controls ( $p = 0.007$ ) (Fig. 2a). This difference did not persist between EE-MSUS and EE-controls ( $p = 0.43$ ) (Fig. 2a).

Overall, these results demonstrate that both MSUS treatment and environmental enrichment alters anxiety levels, and demonstrate that environmental enrichment can abolish the effects of MSUS treatment in F1 animals

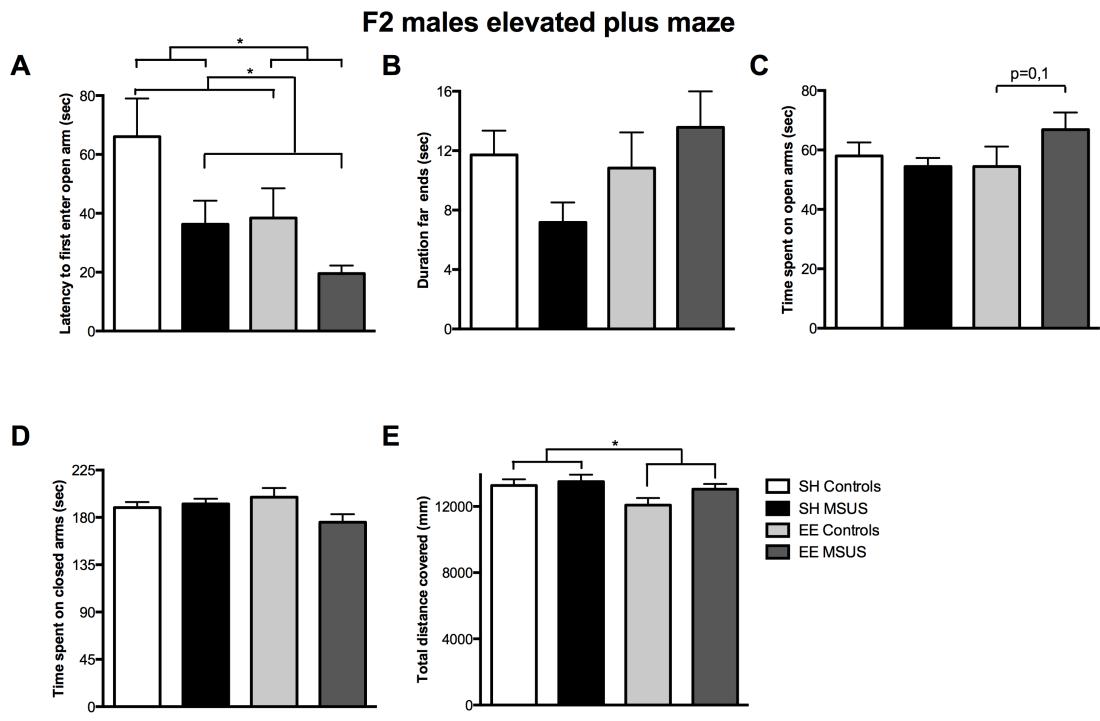
#### **4.4.2. F2 generation**

Next we tested whether paternal exposure to MSUS and/or EE induces behavioral alterations in the normally reared F2 offspring, and whether EE is able to prevent the transmission of MSUS-induced alterations to the next generation.

##### *EPM*

Examining anxiety levels on the EPM in F2 mice by two-way ANOVA showed a significant main effect of stress for the latency to enter the open arms ( $F(1,57) = 4.597$ ,  $p = 0.036$ ), and a significant main effect of housing on the latency to enter the open arms ( $F(1,57)=4.95$ ,  $p=0.03$ ) (Fig.3a) and on the total distance moved ( $F(1,57) = 5.451$ ,  $p = 0.023$ ) (Fig.3e). Further, there was a significant interaction between treatment x housing for the time spent on open arms ( $F(1,57) = 4.177$ ,  $p = 0.046$ ) (Fig.3c) and for the time spent on closed arms ( $F(1,57)= 4.472$ ,  $p=0.039$ ) (Fig.3d), however follow-up post-hoc tests revealed no significant difference between groups on the time spent on open arms (Fig.3c), nor on closed arms (Fig.3d).

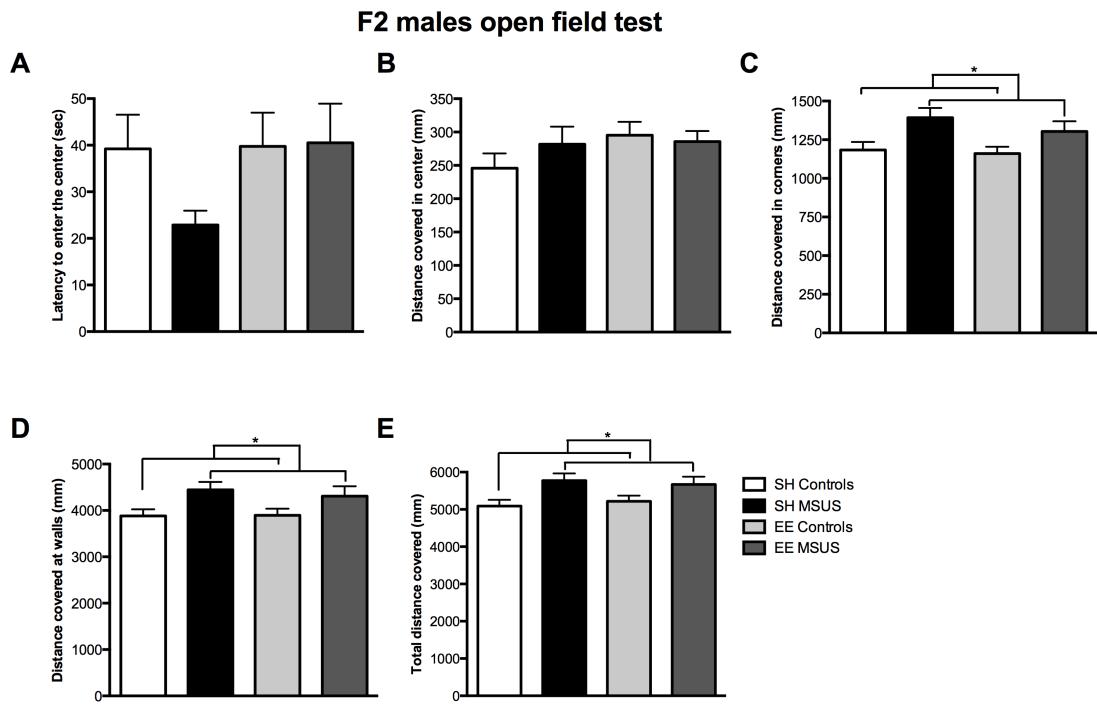
Overall, these results reveal that transgenerational effects of both early life stress and environmental enrichment can be observed on the elevated plus maze in F2 generation mice. Environmental enrichment reduces the latency to enter the aversive open arms, and also reduces locomotor activity on this task. Similarly MSUS treatment reduces the latency to enter the open arms but does not affect locomotor activity.



**Figure 3:** Anxiety-like behaviors in the offspring of mice exposed to MSUS and/or environmental enrichment. (a) latency to first enter an open arm (b) time spent on extreme ends of open arms (c) time spent on open arms (d) time spend on closed arms (e) total distance moved on the EPM.

#### Open field

On the open field test, two-way ANOVA revealed a significant main effect of stress on the distance travelled in the corners ( $F(1,64) = 7.560$ ,  $p = 0.008$ ) (Fig. 3c), distance travelled along the walls of the field ( $F(1,64) = 7.179$ ,  $p = 0.009$ ) (Fig. 4d), and on the total distance travelled ( $F(1,64) = 8.035$ ,  $p = 0.006$ ) (Fig. 4e). There was no significant group effect of housing and no interaction. These results indicate an increase of activity in the offspring of MSUS animals on this task. However, EE has no detectable transgenerational effect on this task.

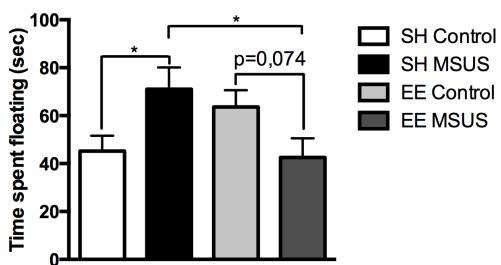


**Figure 4:** Stress sensitivity in the offspring of mice exposed to MSUS and/or environmental enrichment (a) latency to enter the center (b) total distance travelled (c) distance moved in the center (d) distance moved in the corners (e) distance moved at the walls of the open field.

### FST

We showed previously that early life stress also impacts depressive-like behaviors in the offspring (22). We thus examined whether EE can reverse the depressive-like phenotype on the forced swim test (FST) in F2 generation animals. Two-way ANOVA showed a significant interaction of stress x housing ( $F(1,69) = 9.044$ ,  $p=0.004$ ) (Fig.5). Follow-up post-hoc tests revealed that the offspring of SH-MSUS mice spent more time floating than SH-controls ( $p=0.02$ ) and also more time floating than EE-MSUS mice ( $p=0.01$ ). In contrast, the offspring of EE-MSUS mice showed a tendency for a decrease in the time spent floating compared to EE-controls ( $p=0.07$ ). There was no significant difference between the offspring of SH-controls and EE-controls ( $p=0.1$ ). These results suggest that environmental enrichment following early life stress can reverse the stress-induced depressive-like behavior in the offspring.

### F2 males forced swim test



**Figure 5:** Depressive-like behaviors in the offspring of mice exposed to MSUS and/or environmental enrichment (a) time spent floating minutes 3-6 during the FST.

### 4.5. Discussion

Our findings demonstrate that paternal exposure to MSUS and EE can each affect depressive-like and anxiety related behaviors in the offspring. Previous work from our group has demonstrated that fathers exposed to MSUS early in life will transmit depressive-like behavior to their offspring (22), characterized by increased immobility on the forced swim test. The current findings suggest that this transmission can be blocked if the stressed fathers are exposed to EE after weaning (Fig 5).

The ability of EE to reverse stress-induced depressive-like behaviors has previously been described in mice directly exposed to enrichment following early life stress (F1 generation) (168). Similarly, EE was shown to ameliorate depressive-like behaviors in a rat strain bred for learned helplessness, a commonly used rodent model for depression (169). Our findings extend this work by suggesting that a similar reversal of depressive-like behavior by EE can be achieved also across generations. In agreement with our results, Leshem and Schulkin (36) recently also demonstrated that EE following early life stress can counteract several of the negative transgenerational effects of early life stress on the offspring. However, while their study focused on transmission through the female line, our results are the first to demonstrate similar effects through the male line, which is critical as it suggests that the effects can be transmitted through the male germ line and are likely independent of maternal care (127, 167, 170).

While EE was able to transgenerationally reverse the depressive-like phenotype on the forced swim test in MSUS mice, it did not have any effect on time spent floating in the non-stressed controls. Similarly, other studies report no effect of EE on the forced swim test in animals directly exposed to EE (F1 generation) (168, 171), suggesting that EE may only reveal its antidepressant potential in an already compromised system.

The forced swim test is known to be highly sensitive to serotonergic alterations (172), and previous work from our lab has shown that MSUS leads to disruptions in serotonergic signaling across the brain of the offspring (152). Specifically, MSUS offspring show reduced binding to the inhibitory serotonin autoreceptor 5HT1AR in dorsal raphe and hippocampus, and concomitant serotonin hypersecretion in dorsal raphe projection regions. Further, environmental enrichment also impacts the serotonergic system (173-175). EE has been shown to increase 5HT1A receptor binding (176) and can facilitate the antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) (177). Whether these mechanisms underlie the transgenerational effects of EE observed in our study remains to be determined, but it is plausible that due to serotonergic involvement the forced swim test may be particularly sensitive to detect the transgenerational disruptions induced by early life stress, and may thus also be able to unveil the protective effects of EE in our study design. Although the forced swim test is widely used to assess the ability of pharmacologic manipulations on behavioral despair (178, 179), a broader battery of tests will be necessary to fully evaluate the transgenerational interactions between early life stress and environmental enrichment on depressive-like behaviors.

Fathers directly exposed to EE (F1 generation) spent more time in the open arms (Fig.2c) and the far ends of the open arms (Fig.2b), but less time in closed arms (Fig.2d) compared to controls. Further, the latency to enter an open arm was significantly decreased by MSUS in standard housed animals, but this effect did no longer persist in animals exposed to EE (Fig.2a). Despite the reduced latency to enter open arms, MSUS mice overall did not spend more time on the open arms compared to controls. Because time spent on open arms is a widely used measure of anxiety levels (180), this suggests that MSUS mice likely have impairments in risk assessment rather than a gross change in anxiety levels (181). Taken together, our results indicate that in the F1 generation EE has strong anxiolytic effects independent of MSUS, and that EE corrects an impairment in risk assessment induced by MSUS. These findings are consistent with studies in rodents reporting a decrease in anxiety after EE on the EPM (182-186).

In addition to the transgenerational effects on depressive-like behaviors, we also find evidence for transgenerational effects of enrichment and stress on emotional reactivity in the elevated plus maze and open field test. In the elevated plus maze, paternal exposure to MSUS decreased the latency to enter open arms in the offspring of both EE and standard housed animals (Fig.3a) without affecting time spent in open arms, similar to the effects observed in their fathers (Fig.2a). Notably, the latency to enter open arms was substantially different between standard housed control mice in F1

and F2 generation. The reason for this baseline difference is unclear, but is most likely related to the study design; Mice of different generations had to be tested many months apart, possibly introducing confounding variables in the animal care facility as well as subtle differences in the setup of the behavior apparatus that we may not have been able to control for completely. However, the relative effect size between control and MSUS animals in the standard housing condition in F1 and F2 mice is remarkably consistent (about 50%, Fig2a, Fig3a).

Although MSUS seems to affect EPM performance similarly in both generations, the strong anxiolytic effect of EE observed in fathers is not transmitted to offspring, as there is no effect of EE on time spent in open arms in F2 mice. However, paternal EE exposure leads to a decrease in the total distance moved in offspring (Fig.3e), suggesting that under the aversive conditions of the EPM these mice are less actively exploring the maze. This is in contrast to the behavior observed in the fathers directly exposed to EE, who show a strong increase in locomotor activity on the same task (Fig. 2E). Current data suggest that environmental factors (such as MSUS and EE) induce complex epigenetic alterations in the animals directly exposed to the environmental trigger, and that some of these changes can affect the germ line and be passed on to the offspring (167, 187). Consequently, the ensemble of epigenetic alterations induced by environmental conditions may vary considerably between parents and offspring. Furthermore, even if the exact same epigenetic modifications were present in both generations, they will impinge on the organism after birth in the F1 generation, but they will be present starting from conception in the F2 offspring. Therefore, it is not surprising to detect different behavior phenotypes between animals directly exposed to MSUS and their offspring (Gapp et al in revision).

In the open field test, paternal EE seemed to have no overall impact on anxiety behaviors. However, paternal MSUS exposure leads to a specific increase in the distance travelled in the corners and along the walls of the open field (Fig.4c-e). This increase in locomotor activity and thigmotaxis is indicative of increased anxiety. The cause for the different behaviors observed on elevated plus maze and open field test remain unknown, but as the elevated plus maze test is considered to be more aversive than the open field test (188), it is possible that a sufficient level of challenge is necessary to detect effects of EE. As described earlier, a recent report showed that female rats exposed to early life stress and subsequent environmental enrichment transmit behavior alterations to their offspring (36), and the effects detected in the elevated plus maze test are more pronounced than in the open field. Therefore, these tests may not be equally sensitive and may also capture slightly different aspects of anxiety-related behavior.

Several studies have reported on transgenerational transmission of decreased anxiety after EE through alterations in maternal care provided to the offspring (189, 190). In an elegant study, however, Arai and colleagues (35) first demonstrated transgenerational effects of EE on synaptic plasticity and cognitive performance independent of maternal care. Although this effect was transmitted only through females and not through males transmission of the effects persisted also after cross fostering, suggesting that the effects were not due to any alterations in maternal care provided by enriched mothers. However, the transmission could have occurred either through in-utero fetal programming, or through direct, epigenetic alterations in the germ line. If studying the paternal line, in utero effects can be largely ruled out, since males and females are only paired for breeding. Our results thus add to a growing literature describing transgenerational effects of environmental factors in rodent models transmitted through the male line (170, 187). As the sperm cells of F1 males constitute the only direct biological link between F1 and F2 animals, the effects of environmental enrichment on the offspring discovered in our study are most likely due to alterations in the sperm of the enriched F1 males. Previous work from our group has demonstrated that early life stress can alter DNA methylation patterns in the germ line of stressed males (22), and similar effects have been described for other environmental factors such as endocrine disrupters (187) or diet (191). Most of these findings are based on environmental exposures early in life, a time when newborn pups seem to be particularly vulnerable/responsive to external environmental influences (70, 71). However, the germ line stays susceptible to manipulations throughout life, likely involving different epigenetic mechanisms such as non coding RNAs (94, 111, 164) and the retention of histones and their post-translational modifications during spermatogenesis (25, 117). Our current data suggest that alterations in the male germ line may be induced by environmental enrichment during adolescence and/or during adulthood, as the next generation exhibits measurable behavioral alterations.

The fact that early life stress is a potent risk factor for depression in humans, and for depressive-like behavior in rodents, is well accepted (160, 192). However, the possibility that early life stress may also pose a risk to the offspring has only recently been discovered in rodent models (22), but similar findings have already been reported by several independent groups (163, 164). It is not yet clear which other environmental experiences can shape the psychobiological constitution of the offspring through the paternal line. The current findings suggest that in addition to detrimental factors such as traumatic stress, also positive experiences can shape the

behavioral repertoire of the offspring and effectively counteract some of the negative effects induced by stress.

Evidence from human studies suggests that enriched environmental conditions may also exert beneficial effects in children who are at a higher risk of developing neuropsychiatric disease due to adverse conditions in early life. For example, institutional care is characterized by low caregiving quality and social deprivation (193) and children institutionalized at or around birth show delayed social and cognitive development (194) and signs of psychopathology later in life (195). However, when institutionalized children were placed in foster care, they were able to make up for their cognitive delays (196, 197) and had reduced signs of psychopathology (195, 198). Thus, the detrimental long-term effects of deprived environmental conditions early in life may still be counteracted and partially overcome by providing a stimulating environmental setting later in life. Whether beneficial effects of enriched environmental conditions also have transgenerational effects in humans still needs to be determined.

#### **4.6. Acknowledgements**

This work was supported by the Austrian Academy of Sciences, the University Zürich, the Swiss Federal Institute of Technology, Roche, the Swiss National Science Foundation, and The National Competence Center for Research “Neural plasticity and Repair”. J.Bohacek was supported by a postdoc ETHZ fellowship and a Roche fellowship. K. Gapp was supported by a DOC-fFORTE Fellowship from the Austrian Academy of Science. We thank Megan Frugoli for help with the behavioral tests, Heiko Hörster and Francesca Manuella for help with animal caretaking and breeding. The authors report no biomedical financial interests or potential conflicts of interest.

#### **4.7. Authors' contribution**

K.G. designed and performed the experiments, conducted the statistical analyses, interpreted the data and wrote the paper. J.B. designed the experiments, interpreted the results and wrote the manuscript. I.M. supervised the work and wrote the manuscript.

## **5. Implication of sperm RNAs in the inheritance of the effects of early traumatic stress in mice**

Katharina Gapp<sup>1</sup>, Ali Jawaid<sup>1</sup>, Peter Sarkies<sup>2</sup>, Johannes Bohacek<sup>1</sup>, Pawel Pelczar<sup>3</sup>, Julien Prados<sup>4\$</sup>, Laurent Farinelli<sup>4</sup>, Eric Miska<sup>2</sup> and Isabelle M. Mansuy<sup>1\*</sup>

<sup>1</sup> Brain Research Institute, Neuroscience Center Zürich, University of Zürich and Swiss Federal Institute of Technology, Winterthurerstrasse 190, Zürich, CH-8057, Switzerland

<sup>2</sup> Gurdon Institute, Cambridge, United Kingdom

<sup>3</sup> Institute of Laboratory Animal Science, Sternwartstrasse 6; Zürich, CH-8091, Switzerland

<sup>4</sup> FASTERIS SA, Chemin du Pont-du-Centenaire 109, P.O. Box 28, CH-1228 Plan-les-Ouates, Switzerland

\$ Current address: Neuroscience Center, University Geneva, Geneva, Switzerland

\* Corresponding author: mansuy@hifo.uzh.ch

**In revision at Nature Neuroscience**

## **5.1. Summary**

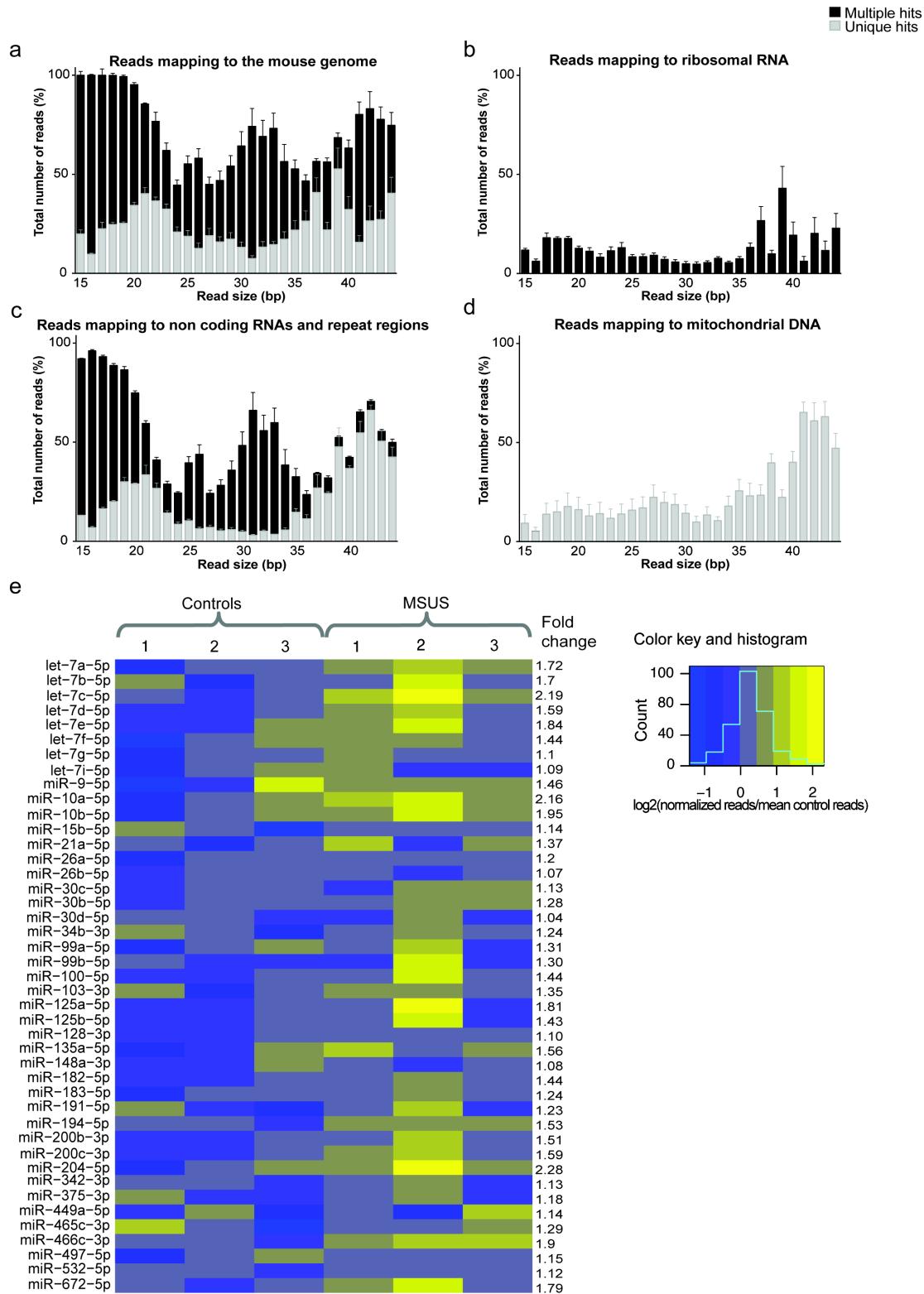
Gene-environment interactions play a key role in the etiology of psychiatric disorders, diabetes and cancer, and are thought to contribute to the inheritance of these diseases across generations. Small non-coding RNAs (sncRNAs) have been proposed as potential vectors at the interface between genes and environment. Here, we report that environmental factors involving traumatic stress in early life in mice affect behavioral responses and glucose metabolism in the progeny, and alter microRNAs (miRNAs) expression. Several miRNAs are altered in adult sperm, and in the serum and brain of both, stressed animals and their adult progeny. Injection of sperm RNAs from stressed males into fertilized wild-type mouse oocytes reproduces the behavioral and metabolic alterations in the resulting offspring. These results suggest that sncRNAs are sensitive to environmental factors in early life, and contribute to the inheritance of stress-induced phenotypes across generations. They may offer potential diagnostic markers for stress pathologies in humans.

## 5.2. Main text

While the genetic make-up of an individual plays a determinant role in the risk to develop a disease, and in disease heritability (199), environmental factors such as early traumatic stress, also play an extremely important role. How they mediate their influence is not fully understood but is postulated to involve epigenetic mechanisms. Small non-coding RNAs (sncRNAs) have been proposed as potential mediators of the interaction between genes and the environment. SncRNAs can relay signals from the environment to the genome and exert regulatory functions on gene activity (200). They have been implicated in gene dysregulation in many diseases including cancer, psychiatric and neurological pathologies, and metabolic disorders (200-202). Recent studies in *C. elegans* (203, 204) and mice (111, 205) have also suggested that sncRNAs can carry or modulate non-Mendelian inheritance, and underlie the transmission of acquired traits induced by external factors. Indeed, sncRNAs are abundant in mature sperm in mammals where they may act as vectors of inheritance of some traits from one generation to the next (206, 207). However, whether sncRNAs in germ cells are influenced by environmental factors such as early stress and contribute to pathological traits associated with these factors has not been determined.

We investigated the involvement of sperm sncRNAs in the impact of traumatic stress across generations, and first examined the content in small RNAs in mouse sperm under normal conditions. Total RNA was prepared from adult sperm, quality-controlled by bioanalyser analyses (Supplementary Fig. 1) then RNAs were sequenced by next-generation deep sequencing (Illumina, Inc). Sequences were collected in a specific range (15-44bp) to increase coverage and quantification accuracy in this range. Sequencing reads were then mapped to the mouse reference genome (UCSC mm9), and to different known populations of sncRNAs, repeat regions and mitochondrial DNA (Fig. 1). These analyses revealed that adult mouse sperm contains several populations of short RNAs of various size that map to the mouse genome to a different degree and fidelity (unique or multiple hits) (Fig. 1a). Approximately 21% of reads mapping to the mouse genome corresponded to typical miRNA size (21-23bp) (Supplementary Fig. 2a) and over 90% of reads mapping to miRNA sequences had a typical miRNA size (Supplementary Fig. 2b). A substantial portion of 26-31bp reads, aligning to piRNA clusters started with the nucleotide T, indicative of piRNAs identity (Supplementary Fig. 2c). Further, a few % of 15-44bp reads also mapped to ribosomal RNAs sequences (Fig. 1b) (short fragments since mRNAs are cleaved in mature

spermatozoa to prevent spurious translation (208) while a significant proportion mapped to other classes of ncRNAs including small cytoplasmic and nuclear RNAs, transfer RNAs, and to repeat regions such as retrotransposon sequences (Fig. 1c). A 16bp read which gave rise to an unusually large peak mapped to a specific piRNA sequence (Supplementary Fig. 3a, b). Finally, reads of all sizes could be mapped uniquely to mitochondrial DNA sequences (Fig. 1d), consistent with the presence of mitochondria in adult sperm (209).



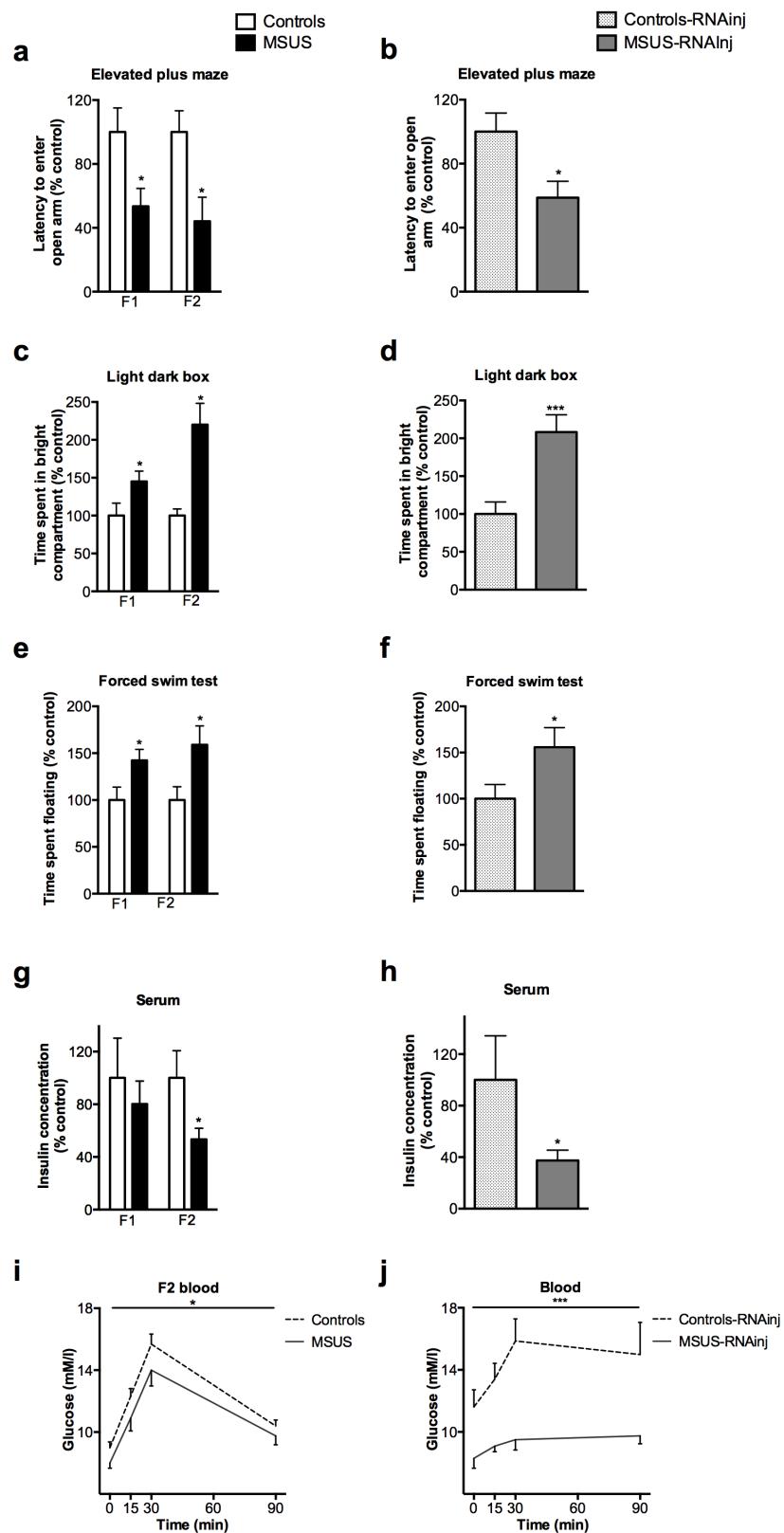
**Figure 1. SncRNAs in adult sperm.** (a-d) RNA composition of sperm from C57Bl/6J adult males. Mapping of 15-44bp sequencing reads to a) the mouse reference genome, b) ribosomal RNAs, c) other non-coding RNAs and repeat regions (repeat masker track from UCSC mm9) and d) mitochondrial DNA, with multiple (mapping to several loci, black) or unique (mapping to a single locus, grey) hits (n=16 mice, pooled in 4 samples). % total reads represents the proportion of reads with a given size mapping to the mouse genome or to selected annotated genomic sequences over the total number of reads of the corresponding size. In a), un-mapped reads may result from PCR pre-sequencing amplification artifacts, incomplete trimming of adapters or sequencing errors, or may reflect the presence of RNA splicing products. In b) a small percentage of reads map to ribosomal RNAs (cleaved) with multiple hits, reflecting ribosomal RNAs cleavage leading to the expected absence of functional ribosomes in sperm cells which are transcriptionally quiescent (208). c) Reads mapping to small cytoplasmic, small nuclear, transfer and low complexity and satellite RNA sequences, signal recognition particle, and to retrotransposon and simple repeats. d) Reads mapping to mitochondrial DNA showing only unique hits allowing unambiguous attribution to mitochondria DNA. This mapping overall reveals the presence of several RNAs populations in mouse sperm, similarly to that observed in human sperm (207). (e) Heatmap showing miRNAs with at least 100 reads in all control libraries that are altered by MSUS in adult sperm (control and MSUS, n=3 samples in each group, each sample containing pooled sperm RNA from 5 mice except control sample 1 which is from one mouse). The blue-to-yellow scale reflects the ratio between the number of normalized reads of a given sample to the mean normalized reads of all control samples for each miRNA.

We next aimed at determining whether the composition of short RNAs in adult sperm is affected by exposure to traumatic stress in early life. We used a model of chronic postnatal maltreatment based on unpredictable maternal separation combined with unpredictable maternal stress (MSUS) in mice (Supplementary Fig. 4) (22, 152, 153). In this model, we first examined the impact of MSUS across generations on behavioral responses by testing adult animals on a battery of tasks. On the elevated plus maze, a test based on the natural avoidance of mice for open and unfamiliar space (210), MSUS males had shorter latency to first enter an open arm than controls (F1, Fig. 2a), that was not due to a change in locomotor activity (Supplementary Fig. 5a), suggesting reduced avoidance and fear. Further in a light-dark box (211), a task that challenges an animal's aversion for brightly lit areas, MSUS males spent more time in

the illuminated compartment than controls (F1, Fig. 2c), suggesting altered response to aversive conditions. Finally, on a Porsolt swim test, a test that challenges behavioral despair (179), MSUS males spent more time floating than controls (F1, Fig. 2e), suggesting depressive-like behaviors. We next examined whether these behavioral alterations could be transmitted to the following generation. F2 offspring were produced by breeding F1 males to wild-type C57Bl/6J females (Supplementary Fig. 4) then when adult, the male F2 offspring were tested on the same behavioral tasks as F1 fathers. On the elevated plus maze, F2 MSUS mice showed a shorter latency to enter an open arm than F2 controls (F2, Fig. 2a), which was not due to altered locomotor activity (Supplementary Fig. 5b). They also spent more time in the bright compartment of the light-dark box than F2 controls, and expressed depressive-like behaviors on the Porsolt swim test (F2, Fig. 2c, e). These results indicate that behavioral traits induced by early traumatic stress are transmitted from one generation to the next.

Since early stress can be a strong metabolic dysregulator (212), we next examined the impact of MSUS on glucose metabolism in our mice, by measuring insulin and glucose. The level of insulin in serum was similar in F1 MSUS and control animals, but was significantly decreased in F2 MSUS progeny (Fig. 2g, non-fasted animals). Blood glucose was also lower in F2 MSUS mice, both at baseline, and during the mounting and recovery of a stress response following an acute restraint stress (Fig. 2i). It was however not affected in F1 animals (Supplementary Fig. 6a). The results showing low glucose level despite low insulin suggest insulin hypersensitivity in F2 animals. To examine this possibility, we conducted glucose and insulin tolerance tests (GTT and ITT respectively) in fasted F1 and F2 males. F1 MSUS males had normal baseline glucose level and glucose clearance on GTT, but had a significantly larger decline in blood glucose after ITT (Supplementary Fig. 6b, c). Likewise, F2 MSUS animals had normal glucose level at baseline, but a significantly lower rise in glucose on GTT (Supplementary Fig. 7a) and normal glucose decrease on ITT except for a slightly better recovery after 120 minutes (Supplementary Fig. 7b). These anomalies on GTT and ITT confirm that glucose metabolism is altered in MSUS animals, possibly due to an insulin hypersensitivity. An overall perturbed basal metabolism was also apparent when measuring body weight and caloric intake. Body weight was lower but caloric intake was increased in F2 MSUS animals, suggesting a hypermetabolism (Supplementary Fig. 7c, d). This was however not observed in F1 MSUS animals (Supplementary Fig. 6d, e). Overall, these results suggest that, in addition to altering behavior, MSUS dysregulates metabolic responses in the offspring of stressed males. The more pronounced effect in the offspring may be due to the fact that alterations are

present already during the first development stages unlike in F1 males, in which MSUS is experienced only after birth.

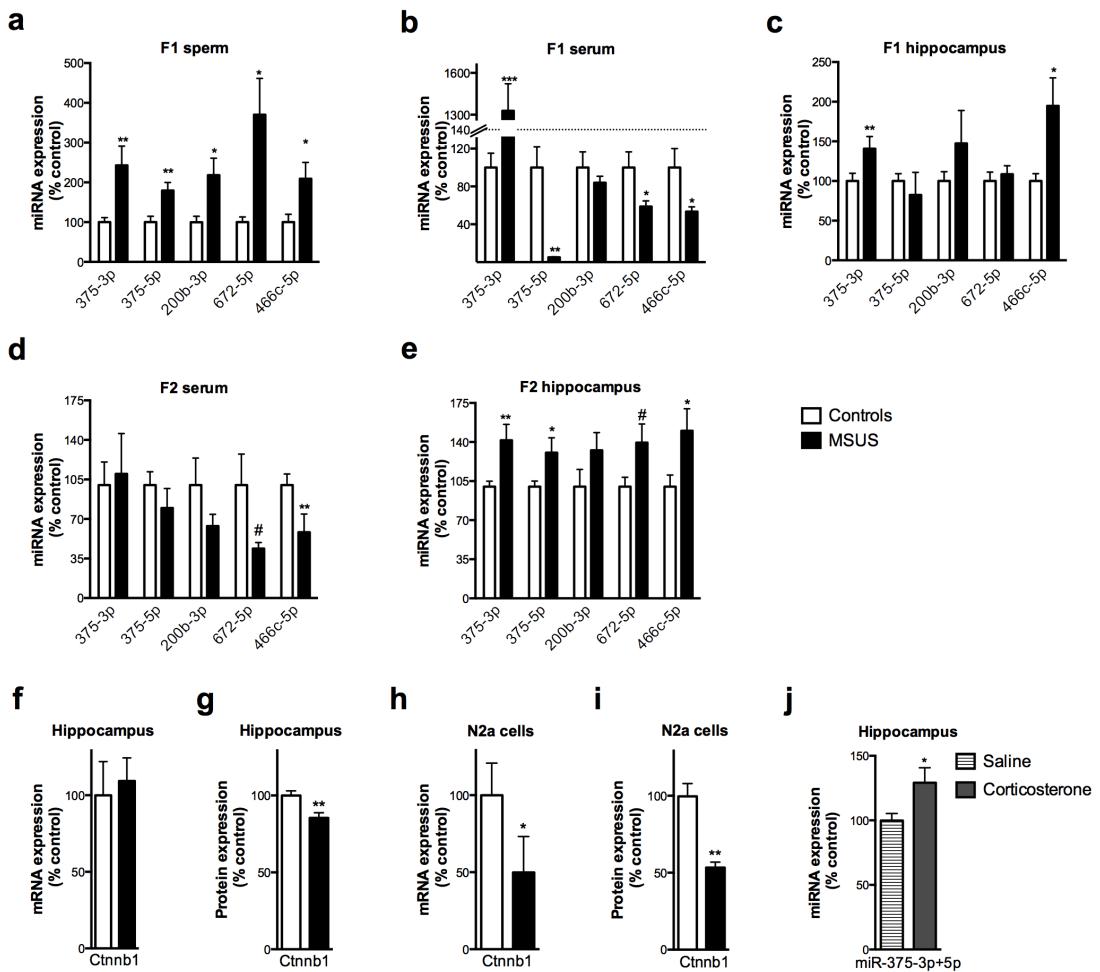


**Figure 2. Behavioral responses in MSUS males across generations and in mice derived from RNA-injected oocytes.** (a, b) Latency to first enter an open arm on an elevated plus maze in a) F1 (control, n=8; MSUS, n=18; t(24)=2.37) and F2 (control, n=30; MSUS, n=25; t(41.98)=3.74) mice, and b) mice from oocytes injected with sperm RNA (RNAinj) from control (Controls-RNAinj, n=19) or MSUS (MSUS-RNAinj, n=20, t(37)=2.67) males. (c, d) Time spent in the bright compartment of the light dark box (c) in F1 (control, n=16; MSUS, n=21; t(35)=-2.14) and F2 (control, n=33; MSUS, n=36; t(41.61)=-3) MSUS mice, and (d) in mice from oocytes injected with sperm RNA from control (Controls-RNAinj, n=15) or MSUS (MSUS-RNAinj, n=17; t(30)=-3.77) males. (e, f) Time spent floating on the forced swim test (e) in F1 (control n=14, MSUS n=16; t(28)=-2.34) and F2 (control n=19, MSUS n=20; t(37)=-2.36) MSUS mice, and (f) in mice from oocytes injected with sperm RNAs from control (Controls-RNAinj, n=18) or MSUS (MSUS-RNAinj, n=20; t(37)=-2.19) males. The results were replicated in two independent experiments. (g, h) Insulin concentration in serum in non-fasted g) F1 (control, n=5; MSUS, n=9; t(12)=0.28) and F2 (control, n=10; MSUS, n=10; t(18)=2.1) MSUS and h) MSUS-RNAinj (Controls-RNA-inj, n=4; MSUS-RNAinj, n=7; t(9)=2.31) males at baseline assessed by Elisa. (i, j) Glucose level in blood in non-fasted i) F2 MSUS (control, n=8; MSUS, n=7; F(1,13)=5.64) and j) MSUS-RNAinj (Controls-RNAinj, n=8; MSUS-RNAinj, n=8; F(1,14)=9.72) males at baseline (time 0) and during the mounting and recovery response after an acute restraint stress (15, 30 and 90 minutes after stress initiation). \*p<0,05, \*\*p<0,01, \*\*\*p<0,001.

In light of the strong behavioral and metabolic symptoms in the offspring of MSUS males, we next investigated whether MSUS has an effect on sperm sncRNAs. We compared small RNA sequences in sperm of adult F1 MSUS and control males by deep sequencing. These analyses revealed that the expression of several miRNAs was upregulated in the sperm of MSUS males (Fig. 1e). Determining predicted targets of these miRNAs using the prediction algorithm Diana microT CDS (213) identified several potential candidate genes (73 in total), some which are targeted by one miRNA, others by multiple miRNAs. Notably, several of these targets are implicated in DNA/RNA regulation, in epigenetic regulation or RNA binding and processing (Supplementary Table 1). The analyses also showed that, in addition to miRNAs, piRNAs were also affected by MSUS. Cluster 110 on chromosome 13 in particular, showed a downregulation in MSUS sperm compared to controls (Supplementary Fig. 8), suggesting a potential implication of piRNAs in the effects of MSUS.

We next validated the deep sequencing data by examining the expression level of selected miRNAs in MSUS and control animals by reverse transcription quantitative real time PCR (RT-qPCR). These analyses confirmed that miR-375-3p, the corresponding 5p leading strand miR-375-5p, miR-200b-3p, miR-672-5p and miR-466-5p were up-regulated in sperm from adult MSUS males (by about 143% for miR-375-3p, 80% for miR-375-5p, 109% for miR-200b-3p, 270% for miR-672-5p and 121% for miR-466c-5p) (Fig. 3a), corroborating the deep sequencing data. We also examined if the miRNAs are altered in other adult tissues/cells than sperm. Several of the miRNAs were up-regulated in serum, as well as in the hippocampus and hypothalamus, brain structures involved in stress response (214), in adult F1 MSUS animals (Fig. 3b, c and Supplementary Fig. 9a). Other brain structures like cortex did not show an alteration of these miRNAs (Supplementary Fig. 9b). Since miRNAs were affected in sperm in F1 MSUS males, we next examined whether they were also changed in the F2 progeny. Strikingly, several miRNAs were significantly altered in the serum and hippocampus of adult F2 MSUS mice (Fig. 3d, e), suggesting a transmission of the alterations from father to offspring. No change in F2 sperm could be detected (Supplementary Fig. 10), despite the fact that F2 MSUS males can transmit the behavioral phenotype to F3 animals (22). This suggests the possibility that miRNAs changes may be transferred to epigenetic marks, perhaps involving DNA methylation and/or histone post-translational modifications, for long-term maintenance.

To investigate the functional outcome of miRNAs alteration, we examined potential targets of one of the affected miRNAs, miR-375, since it has functions in stress response (215) and metabolic regulation (216) and neuronal differentiation (217). We observed that catenin  $\beta$ 1 (Ctnnb1), a predicted miR-375 target implicated in mood disorders (218) and known to be down-regulated after chronic stress (219), was decreased in F2 male hippocampus (Fig. 3f, g). To confirm the relationship between miR-375 and Ctnnb1, we carried out *in vitro* transfection assays in mouse neuronal cultures. Expression of a miR-375-3p mimic significantly down-regulated Ctnnb1 (Fig. 3h, i), suggesting a direct effect of miR-375 on Ctnnb1. We also examined the link between miR-375 and stress and tested the effect of corticosterone on miR-375 expression *in vivo*. Injection of corticosterone in adult wild-type males significantly increased miR-375 expression in the hippocampus (Fig. 3j), confirming a relationship between this miRNA and stress.



**Figure 3. Molecular effects of MSUS in adult F1 and F2 mice.** (a-f) Expression analyses of candidate miRNAs in (a) sperm of F1 control and MSUS adult males (miR-375-3p: control, n=10; MSUS, n=9; t(7.679)=-2.79. miR-375-5p: control, n=10; MSUS, n=10; t(18)=-3.19. miR-200b-3p: control, n=10; MSUS, n=10; t(11.17)=-2.46. miR-672-5p: control, n=10; MSUS, n=10; t(9.38)=-2.92. miR-466c-5p: control, n=10; MSUS, n=10 t(13.05)=-2.4), (b) serum of F1 control and MSUS adult males (miR-375-3p: control, n=8; MSUS, n=8; t(7.06)=-5.17. miR-375-5p: control, n=8; MSUS, n=8; t(7.01)=4.33. miR-200b-3p: control, n=8; MSUS, n=7; t(9.3)=0.90. miR-672-5p: control, n=8; MSUS, n=8; t(8.8)=2.24. miR-466c-5p: control, n=8; MSUS, n=7; t(7.90)=2.26), (c) hippocampus of F1 control and MSUS adult males (miR-375-3p: control, n=8; MSUS, n=6; t(12)=-2.34. miR-375-5p: control, n=8, MSUS, n=6; t(6.045)=0.59. miR-200b-3p: control, n=8; MSUS, n=6; t(5.8)=-1.1. miR-672-5p: control, n=8; MSUS, n=6; t(12)=-0.54. miR-466c-5p: control, n=8; MSUS, n=6; t(11)=-2.79), (d) serum of F2 control and MSUS adult males (miR-375-3p: control, n=6; MSUS, n=6; t(9)=0.93. miR-375-5p: control, n=5; MSUS, n=6; t(9)=0.93. miR-200b-3p: control, n=6; MSUS, n=6; t(10)=1.38. miR-672-5p: control, n=6; MSUS, n=6; t(5.29)=2.08. miR-466c-5p: control, n=6; MSUS, n=6; t(10)=2.21), (e), hippocampus of

F2 control and MSUS adult males (miR-375-3p: control, n=7; MSUS, n=8; t(8.62)=-2.74. miR-375-5p: control, n=14; MSUS, n=15; t(17,89)=-2.14. miR-200b-3p: control, n=8; MSUS, n=8; t(14)=-1.47. miR-672-5p: control, n=7; MSUS, n=8; t(13)=-2.01. miR-466c-5p: control, n=7; MSUS, n=8; t(13)=-2.15). (f, g) Level of *Ctnnb1* (f) mRNA (control n=7; MSUS n=7; t(12)=0.4) and (g) protein (control n=7; MSUS n=6; t(11)=3.26) in hippocampus of F2 control and MSUS males. (h, i) *Ctnnb1* (h) mRNA (control n=3; transfected n=3, t(4)=2.78) and (i) protein (control n=3; transfected, n=3 t(4)=5.14) in N2a cells transfected with miR-375-3p mimic. (j) Level of miR-375-3p+5p in the adult hippocampus after acute injection of corticosterone (Saline n=14, corticosterone n=14; t(26)=2.27). Sequencing results were validated by RT-qPCR on the same samples as those used for deep sequencing. The results were replicated in an independent experiment using samples from a different batch of animals. #p<0.1, \*p≤0.05, \*\*p<0.01, \*\*\*p≤0.001.

Finally, to provide more direct evidence that sperm RNAs are potential mediators of the effect of MSUS from one generation to the next, sperm RNA from adult control and MSUS males was microinjected into wild-type fertilized mouse oocytes and the resulting offspring (Controls-RNAinj and MSUS-RNAinj respectively) were analyzed behaviorally when they had reached adulthood. On the elevated plus maze, MSUS-RNAinj animals had a lower latency to first enter an open arm than controls-RNAinj animals (Fig. 2b), that was not due to any change in locomotor activity (Supplementary Fig. 5c). In the light dark box, they spent more time in the bright compartment, and they floated longer in the forced swim test than controls-RNAinj (Fig. 2d,f). These results indicate an overall similar behavioral profile to that observed in MSUS animals. Further, metabolic analyses showed that the level of insulin, and of glucose at baseline and after acute stress was lower in MSUS-RNAinj animals than in controls-RNAinj (Fig. 2h, j). MSUS-RNAinj animals also had a trend for lower body weight than controls-RNAinj animals (Supplementary Fig. 11a). Interestingly, miR-375-5p was upregulated in the hippocampus of MSUS-RNAinj animals, similar to that in the hippocampus of F2 MSUS animals derived from natural breeding (Supplementary Fig. 11b). Overall, these results suggest that comparable behavioral, metabolic and molecular effects are induced by direct exposure to MSUS and by oocyte-injection of sperm RNAs from males exposed to MSUS.

Together, these results provide evidence that traumatic stress experienced by males in early postnatal life can affect miRNAs expression in germ cells and other tissues/cells including serum and brain, and alter behavioral and metabolic responses

in these males and their offspring. The results further show that sperm RNAs from stressed males injected into wild-type oocytes can reproduce some of the behavioral and metabolic symptoms in the resulting animals, suggesting that the transmitted effects of early traumatic stress implicate small RNAs. The findings provide new evidence in mammals that RNA-dependent processes are likely involved in the transmission of acquired traits. They significantly extend previous findings in *C. elegans* and *Drosophila* demonstrating the contribution of sncRNAs in epigenetic inheritance (44-46). These findings also corroborate previous reports that specific miRNAs are altered in the brain by acute or chronic stress (212, 220-222) but now show that several miRNAs can be affected in different tissues and across generation. The mechanisms underlying the behavioral and metabolic alterations remain mostly undefined, but likely implicate multiple genes and molecular pathways during development and adulthood. The identification of potential miRNAs targets involved in epigenetic regulation, transcriptional control, DNA/RNA binding and processing reflects such complexity. Among the miRNAs affected by postnatal traumatic stress, miR-375 may be an important player in the brain considering its role in the regulation of pro-opiomelanocortin (POMC)-CRF pathways(223), and the implication of its target Ctnn1b in Wnt signaling and neuronal functions (224). miR-375 is also implicated in pancreatic functions; it is stimulated by glucose in humans and mice (215) and negatively regulates glucose-induced insulin secretion (216). Other miRNAs found to be altered in sperm also surely contribute to the phenotypes. Finally, these findings newly highlight the sensitivity of sncRNAs in germ cells to stress, and the consequences that exposure to chronic stress in early postnatal life can have on sncRNAs. By extension, these findings are expected to help the identification of molecular markers of traumatic stress, and the potential diagnostics of stress-induced disorders and stress predisposition in humans.

### **5.3. Acknowledgements**

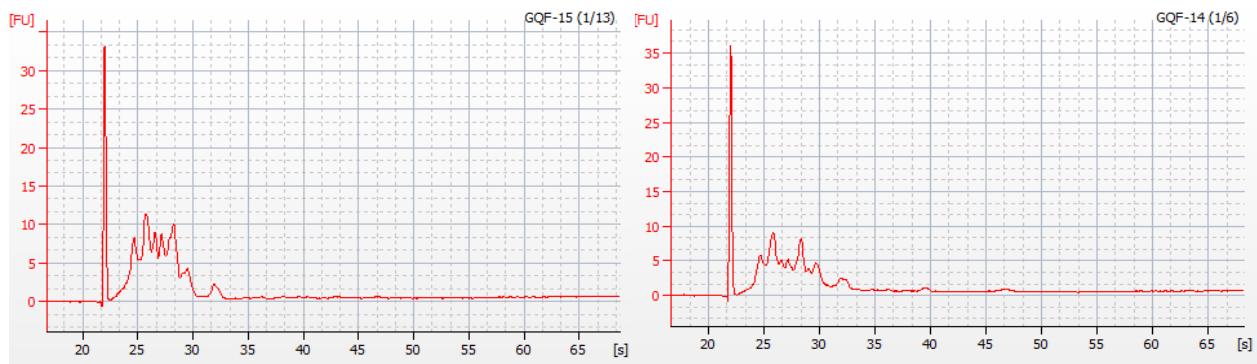
This work was supported by the Austrian Academy of Sciences, the University Zürich, the Swiss Federal Institute of Technology, Roche, the Swiss National Science Foundation, and The National Competence Center for Research “Neural Plasticity and Repair”. P.S. was supported by a Gonville and Caius College fellowship. We thank Minoo Razoulzadegan and Valérie Grandjean for help with the sperm purification, Francesca Manuella and Heiko Hörster for assistance with the MSUS paradigm, Hans Welzl for help with behavior, Grégoire Vernaz for help with Western blotting, and Alon Chen and Andrea Brunner for constructive discussions.

#### **5.4. Authors' contribution**

K.G. did all RT-qPCRs, behavioral tests, metabolic measurements, sperm RNA preparation for sequencing libraries construction and for RNA injection into fertilized oocytes and part of the sequencing analyses. A.J. performed Western blots and cell culture experiments and assisted with metabolic measurements. J.B. carried out the MSUS procedures and produced MSUS animals. J.P. and P.S. did most RNA sequencing analyses. P.P. carried out the RNA injection experiments. E.M. and L.F. helped design RNA sequencing analysis. K.G. and I.M.M. designed the study, interpreted the results and wrote the manuscript.

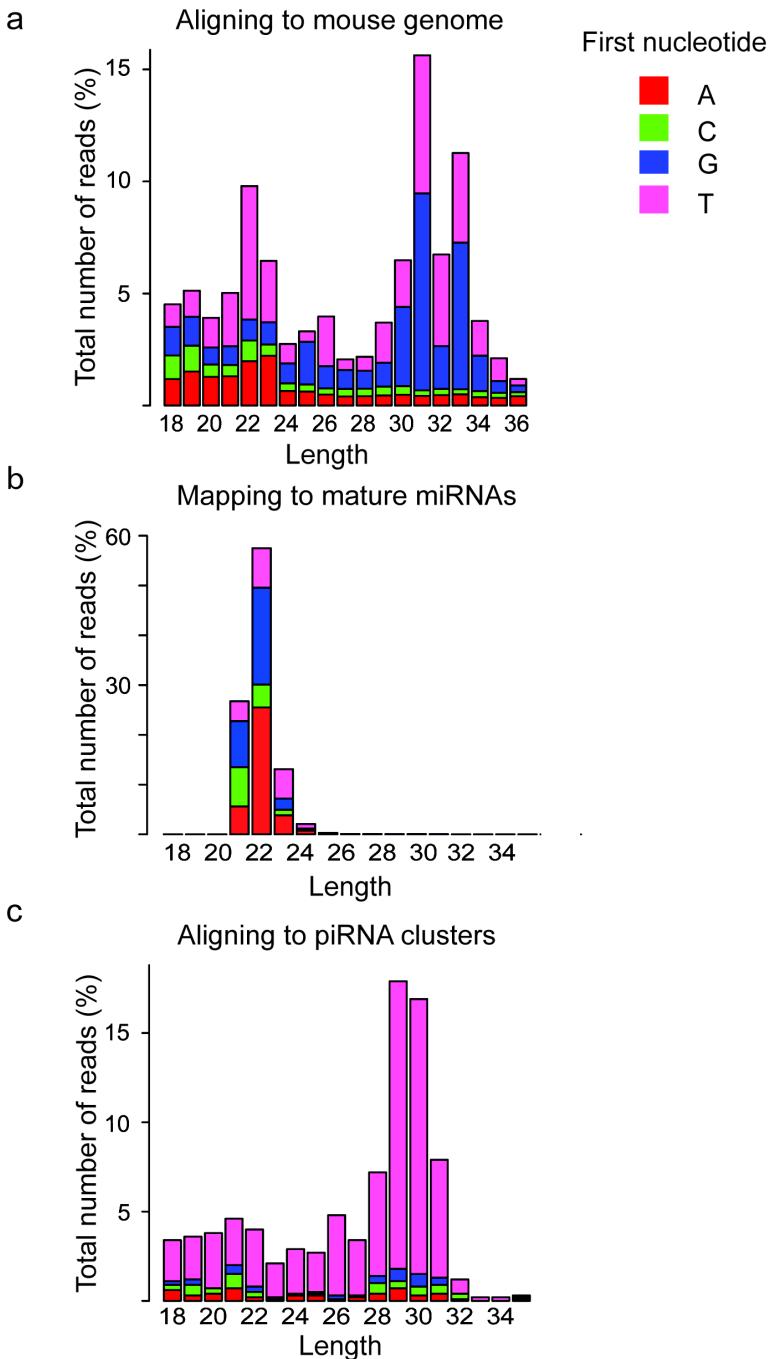
## 5.5. Supplementary Material

**Supplementary Figure 1**



**Size and integrity profiles of sperm RNAs used for deep sequencing and injected into fertilized oocytes by bioanalyzer analyses.** Representative electropherograms show fluorescence intensity (fluorescence unit, FU) over time (seconds) during pulsing of an RNA sample through a separation microchannel in the bioanalyzer. Small RNAs go through the microchannel faster than long RNAs and should appear on the left of the x-axis (for instance, 25bp RNAs should appear after about 23 seconds, 200bp RNAs after 28 seconds and 2kb RNAs around 44 seconds). GQF-15 corresponds to a control sample and GQF-14 to a MSUS sample (pooled RNA from 5 mice). Both samples appear to contain short RNAs but no apparent long RNAs.

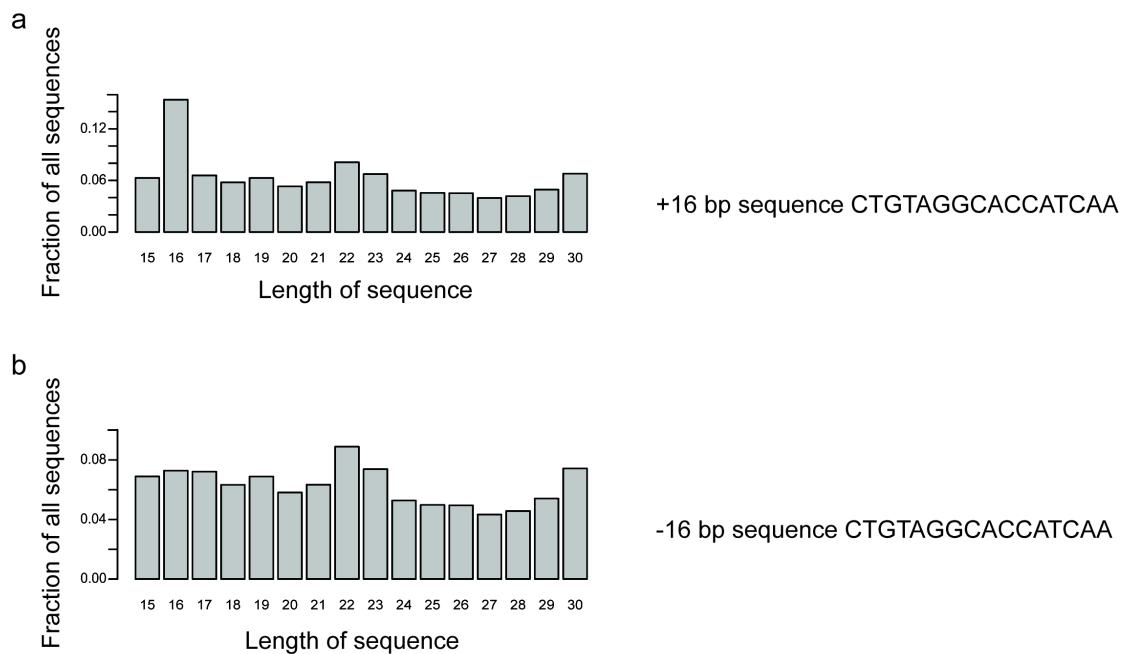
## Supplementary Figure 2



**Illustration of short RNA reads** (a) aligning to the mouse genome, (b) mapping to mature miRNA sequences (allowing for overhanging 5' and 3' nucleotides) and (c) aligning to piRNA clusters. In (a), reads alignment shows peaks at the typical size of miRNAs (21-23bp) and piRNAs (26-31bp). In (b), mapping of 18-35bp reads that do not map to the transcriptome, to annotated miRNAs shows a sharp peak at 22bp, the typical size of mature miRNAs. In (c), alignment of 18-35bp reads that do not map to the

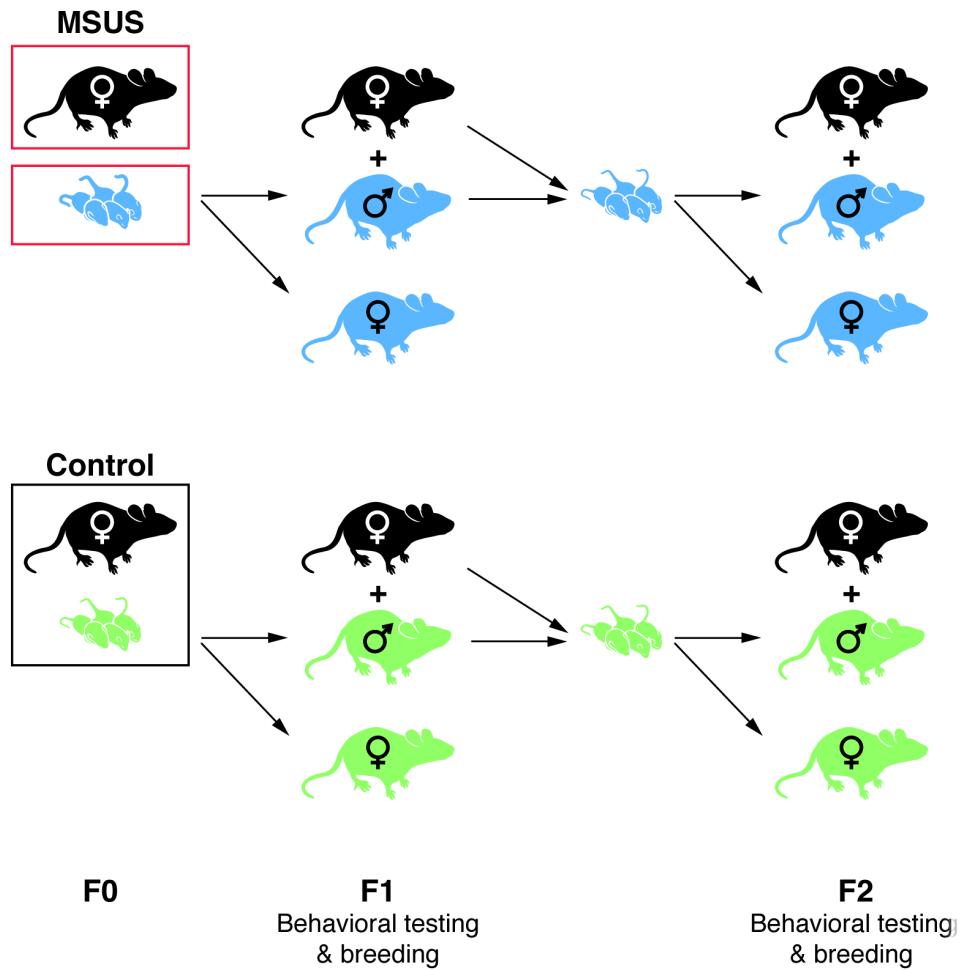
transcriptome, to genomic regions annotated as piRNAs shows a peak at the typical size of piRNAs, starting with the nucleotide T indicative for a piRNA identity. A concatenation of all reads detected in control libraries is shown. The size and first nucleotide are shown by position on the x-axis and color, respectively. The y-axis shows the percentage of reads count relative to total reads count for the combined libraries.

### Supplementary Figure 3



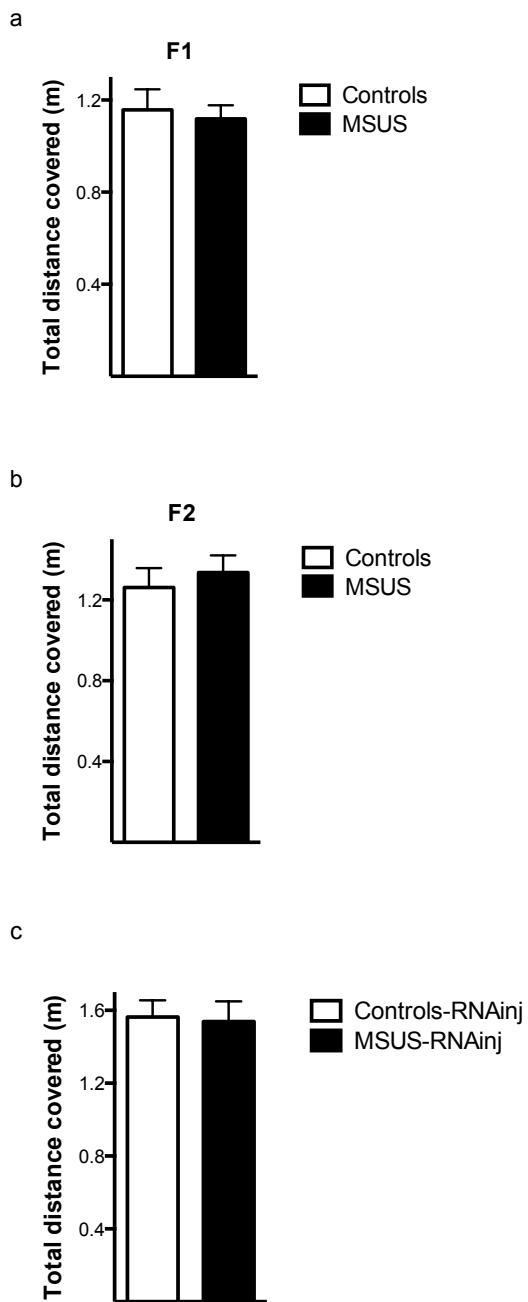
**Proportion of 15-30bp reads including or excluding a 16bp specific sequence.** The y-axis represents the percentage of reads relative to total reads of combined control libraries either a) including an abundant 16bp sequence corresponding to an annotated piRNA sequence or b) excluding this 16bp sequence. Exclusion of this sequence results in a loss of the apparent enrichment of the 16bp peak, suggesting that the peak is not an artefact. A concatenation of all reads detected in control libraries is shown.

**Supplementary Figure 4**



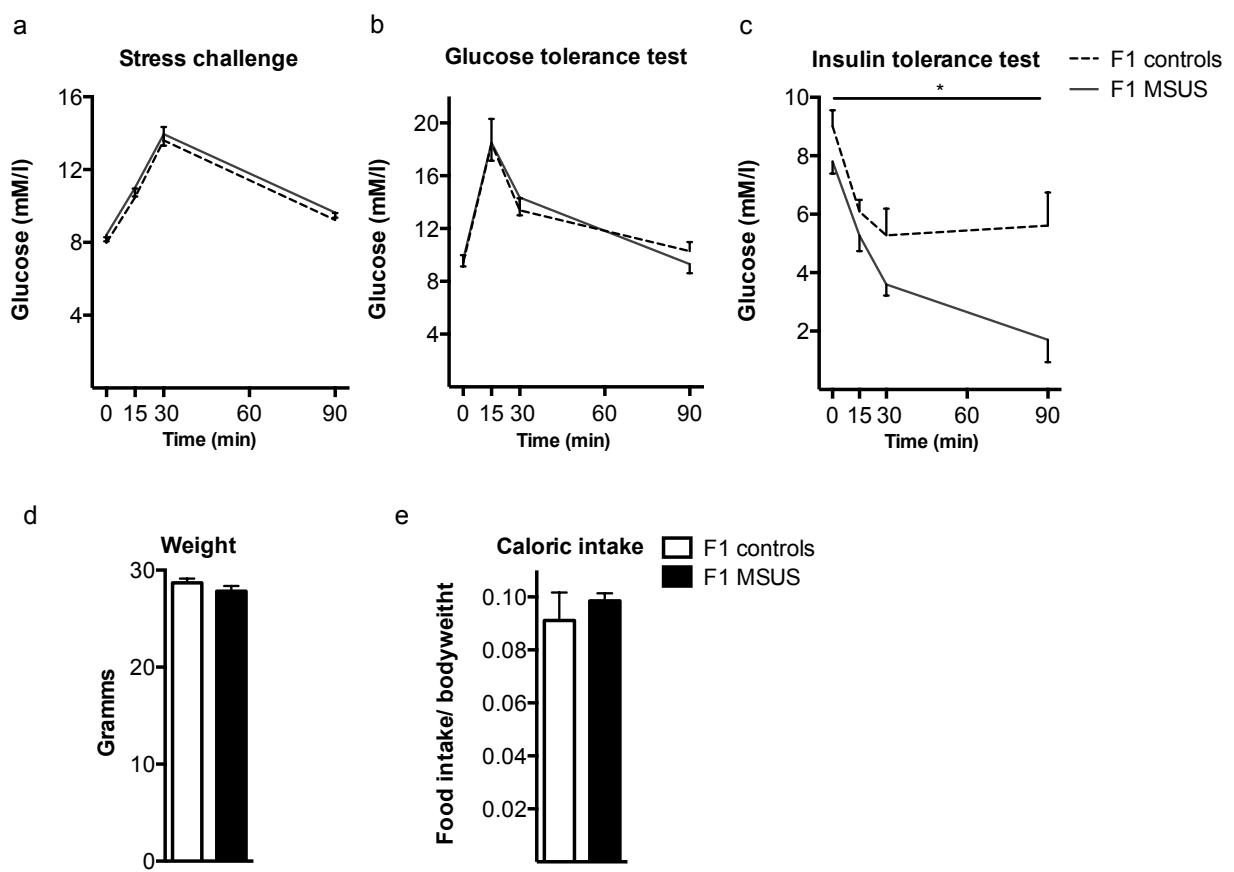
**Experimental design of MSUS treatment and breeding.** C57Bl/6J F0 females (left) bred to C57Bl/6J males were subjected to MSUS from postnatal day (PND) 1 to 14 or allowed to raise their offspring in normal conditions (Control). Males from the F1 offspring were then bred to naïve C57Bl/6J females to obtain second-generation animals (F2) that were raised in normal conditions (no maternal separation or maternal stress).

**Supplementary Figure 5**



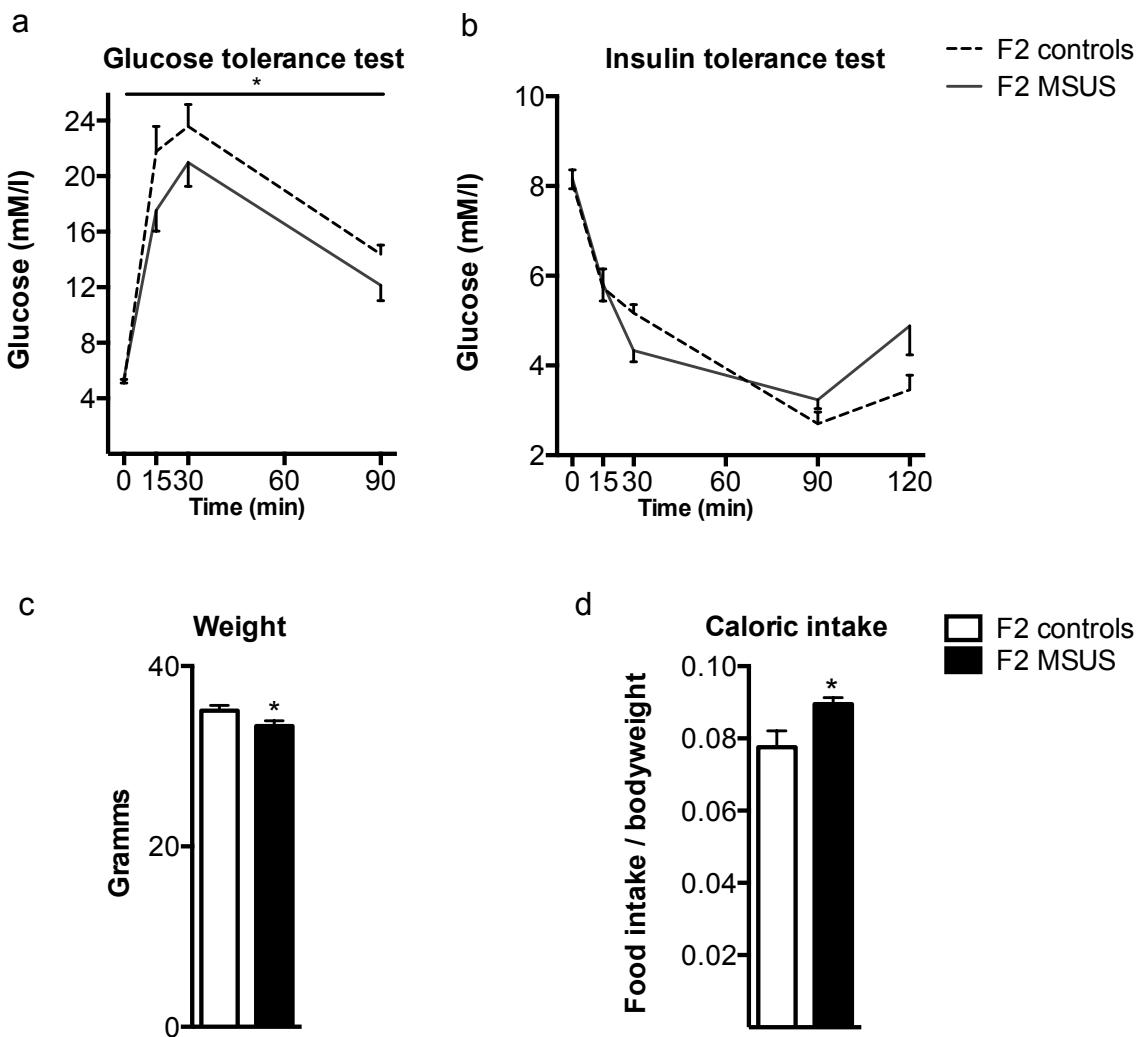
**Activity on an elevated plus maze.** Total distance covered by adult (a) F1 (controls n=8, MSUS n=17;  $t(23)=0.55$ ), (b) F2 (controls n=30, MSUS n=30;  $t(53)=-1.06$ ) and (c) RNA-injected (controls-RNAinj n=18, MSUS-RNAinj n=19,  $t(35)=0.18$ ) animals. Data are mean  $\pm$  s.e.m.

## Supplementary Figure 6



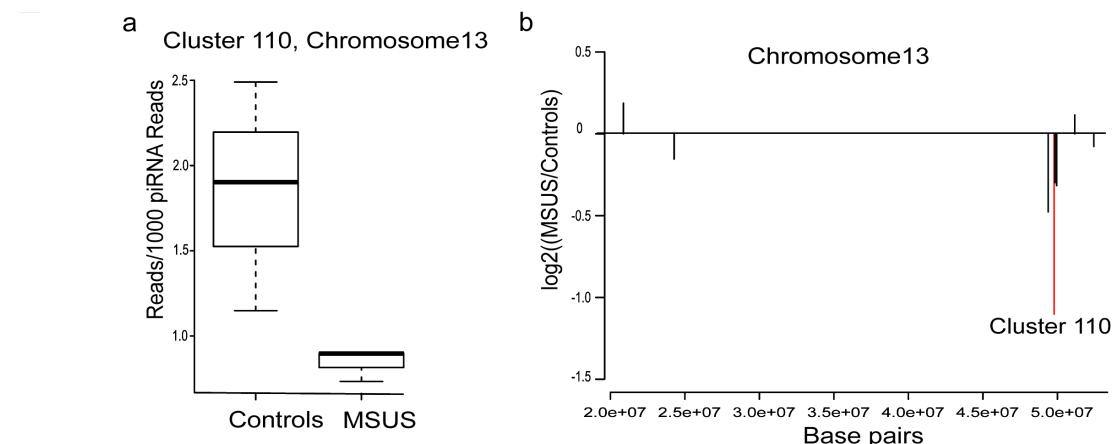
**Metabolic profile in F1 MSUS animals** (a-c) Glucose level in blood at a) baseline and during the mounting and recovery response after an acute restraint stress in non-fasted F1 mice (control, n=8; MSUS, n=8;  $F(1,22)=4.26$ ) b) at baseline and during the mounting and recovery in a glucose tolerance test (GTT) in fasted F1 mice (control, n=8; MSUS, n=8;  $F(1,14)=0.01$ ) c) at baseline and during the decrease and recovery in an insulin tolerance test (ITT) in fasted F1 mice (control, n=8; MSUS, n=6;  $F(1,12)=5.38$ ). (d) Body weight (control, n=10; MSUS, n=13;  $t(21)=1.82$ ) and (e) caloric intake (control, n=4; MSUS, n=6;  $t(8)=-0.81$ ) in F1 adult animals. Data are mean  $\pm$  s.e.m. \* $p<0.05$

**Supplementary Figure 7**



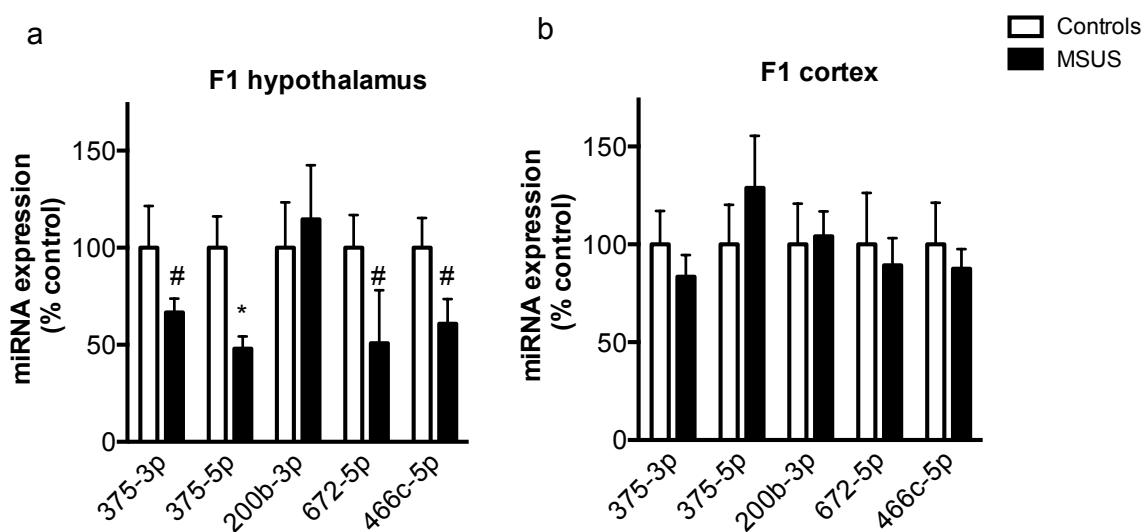
**Metabolic profile in F2 MSUS animals** (a-b) Glucose level in blood at (a) at baseline and during the mounting and recovery phase after an acute glucose challenge in fasted F2 mice (control, n=8; MSUS, n=8;  $F(1,14)=4.71$ ) and b) at baseline and during the decrease and recovery phase after an acute insulin challenge in fasted F2 mice (control, n=7; MSUS, n=6;  $F(4,44)=3.38$ ; 0 min:  $t(11)=-2.5$ , 15 min:  $t(11)=-0.15$ , 30 min:  $t(11)=2.76$ , 90 min:  $t(11)=-1.58$ ). (c) Body weight (control, n=13; MSUS, n=11;  $t(21)=2.09$ ) and (d) caloric intake (control, n=6; MSUS, n=6;  $t(6.52)=-2.44$ ) in F2 adult animals. Data are mean  $\pm$  s.e.m, \* $p<0.05$  group effect repeated measures ANOVA and t-test.

## Supplementary Figure 8



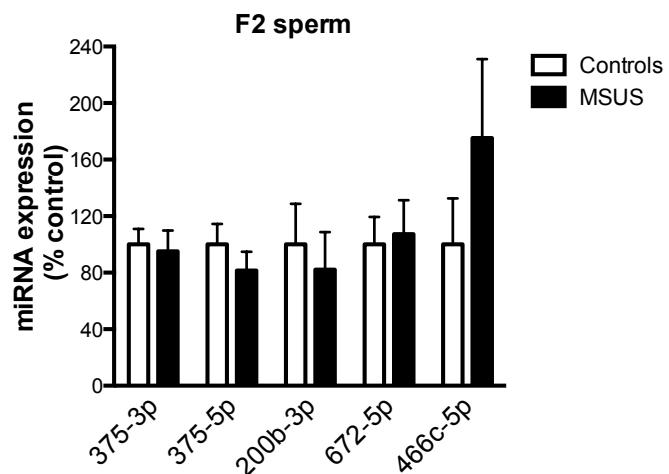
**Effect of MSUS on piRNAs in adult sperm.** (a) Boxplot showing reads aligning to piRNA cluster 110 (on chromosome 13) per 1000 piRNAs reads in control and MSUS samples (negative binomial test  $p<0.1$  after Bonferroni multiple test correction). (b) Log2 of the ratio of MSUS to control reads aligned to piRNA clusters on chromosome 13 showing that cluster 110 (red) is downregulated and is surrounded by two other clusters which are also downregulated in MSUS samples.

### Supplementary Figure 9



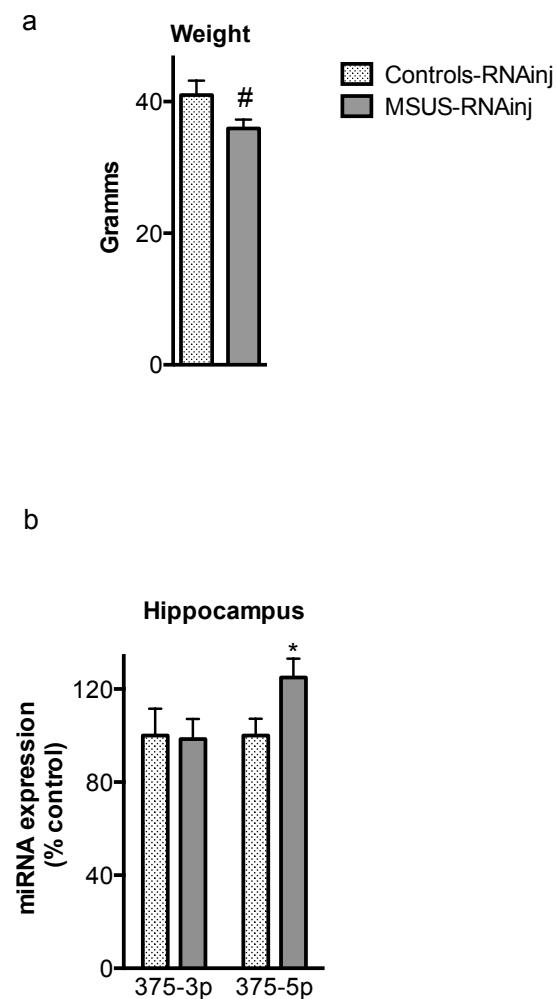
**MiRNA expression in hypothalamus and cortex in F1 mice.** Level of miRNAs expression in (a) hypothalamus (miR-375-3p: control, n=3; MSUS, n=4; t(5)=1.68; miR-375-5p: control, n=3; MSUS, n=4; t(5)=3.38; miR-200b-3p: control, n=3; MSUS, n=4; t(5)=-0.38; miR-672-5p: control, n=3; MSUS, n=4; t(6)=2.02; miR-466c-5p: control, n=3; MSUS, n=4; t(5)=1.98) and (b) cortex (miR-375-3p: control, n=4; MSUS, n=4; t(6)=0.81; miR-375-5p: control, n=4; MSUS, n=4; t(6)=-0.86; miR-200b-3p: control, n=4; MSUS, n=4; t(6)=-1.17; miR-672-5p: control, n=4; MSUS, n=4; t(6)=0.36; miR-466c-5p: control, n=4; MSUS, n=4; t(6)=0.53) of adult F1 MSUS males. Data are mean  $\pm$  s.e.m, \*p<0,05, #p<0,1.

### Supplementary Figure 10



**MiRNAs expression in F2 sperm.** Similar miRNA expression in control and MSUS adult males (miR-375-3p: control, n=8; MSUS, n=8; t(14)=0.26; miR-375-5p: control, n=8; MSUS, n=8; t(14)=0.94; miR-200b-3p: control, n=4; MSUS, n=3; t(5)=0.44; miR-672-5p: control, n=4; MSUS, n=4; t(6)=-0.24; miR-466c-5p: control, n=4; MSUS, n=4; t(6)=-1.16).

**Supplementary Figure 11 (Gapp et al)**



**Analyses in MSUS-RNAinj males** (a) Body weight of adult control-RNAinj (n=8) and MSUS-RNAinj (n=9) animals ( $t(15)=1.9$ ). (b) Level of miR-375-3p (Controls-RNAinj n=7, MSUS-RNAinj n=8;  $t(13)=.10$ ) and miR-375-5p (controls-RNAinj n=7, MSUS-RNAinj n=7;  $t(12)=-2.3$ ) in the adult hippocampus. Data are mean  $\pm$  s.e.m, \* $p<0,05$ , # $p<0,1$ .

**Supplementary table 1**

Gene name	miRNA																										
	let-7-5p	9-p5	10a-5p	15b-5p	21a-5p	26a-5p	30b-5p	34b-5p	99a-5p	100-5p	103-3p	125a-5p	128-3p	135a-5p	148a-3p	182-5p	183-5p	194-5p	200b-3p	204-5p	342-3p	375-3p	449a-5p	465c-3p	466c-3p	497-5p	532-5p
ACSL4																											
ACVR2A																											
AI314180																											
AK220484																											
AQP11																											
ASH1L*																											
BC030336																											
BCL2																											
CADM2																											
CPEB2 *																											
DCX																											
DYRK1B																											
E2F3 *																											
EIF2C4 *																											
EIF4E2 *																											
ELAVL4																											
ELL2 *																											
ESRRG																											
FMR1																											
FOXN2 *																											
FRS2																											
GMFB																											
HS3ST3B1																											
IL1RAPL1																											
INHBB																											
INO80D *																											
JARID2 *																											
LIN28 *																											
LIN28B *																											
MACF1																											
MAP3K1																											
MMD																											
MSL2 *																											
N4BP1 *																											
NARG1																											
NFIA *																											
NR4A2 *																											
NTNG1																											
ODZ2																											
OTUD4																											
PCMT1																											
PNPLA6																											
PPARGC1B																											
PRTG																											
PTPRD																											
PWWP2B *																											

	miRNA																										
Gene name	let-7-5p	9-p5	10a-5p	15b-5p	21a-5p	26a-5p	30b-5p	34b-5p	99a-5p	100-5p	103-3p	125a-5p	128-3p	135a-5p	148a-3p	182-5p	183-5p	194-5p	200b-3p	204-5p	342-3p	375-3p	449a-5p	466c-3p	497-5p	532-5p	672-5p
RASGEF1B																											
RC3H2			■																								
RFX5 *				■																							
RNF138																	■										
RNF38																			■								
RORA *		■																									
RSPO3				■																							
SALL1 *											■							■									
SEMA3A						■																					
SIRT1 *	■																										
SLC8A1				■																							
SOCS6					■																						
SOX6 *						■																					
TAOK1																											
TLK1 *			■								■																
TNRC6B *				■			■																				
UBR3 *								■																			
WEE1									■																		
WIP12										■							■										
ZBTB34 *											■																
ZBTB39 *					■																						
ZFHX4 *																											
ZFP462 *						■																					
ZFP711*											■																
ZFPM2 *												■															
ZFYVE26 *	■																										

**Predicted targets of miRNAs with altered expression in MSUS sperm.** Common predicted target genes shared by 3 or 4 miRNAs are indicated by gray boxes. Genes targeted by different miRNAs from the same family are counted as one. \* highlights genes with a functional implication in epigenetic regulation, i.e. the histone deacetylase SIRT1, transcriptional control (transcription factors), DNA/RNA binding (zinc-finger proteins), and DNA synthesis and repair (DNA polymerases).

## **5.6. Supplementary methods**

### **5.6.1. Animals**

C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature and humidity-controlled facility with food and water *ad libitum*. All experimental manipulations were performed during the animals' active cycle in accordance with guidelines and regulations of the cantonal veterinary office, Zurich.

### **5.6.2. Mice treatment**

For unpredictable maternal separation combined with maternal stress (MSUS), C57Bl/6J dams (2-3 months-old) and litters were subjected to daily 3hr proximal separation from postnatal day 1 to 14 as described previously (22). Control animals were left undisturbed apart from a cage change once a week until weaning (postnatal day 21). Once weaned, pups were reared in social groups (4-5 mice/cage) composed of animals subjected to the same treatment but from different dams to avoid litter effects. To obtain second and third generations, adult F1 males (5 months old) were bred with naïve C57Bl/6J females.

### **5.6.3. Preparation of sperm samples**

Mature sperm cells extracted from cauda epididymis from males were separated from somatic cells by counterflow centrifugal elutriation using a Beckman JE-5.0 elutriation rotor in a Sanderson chamber and a Beckman Avanti J-26 XPI Elutriation Centrifuge. Briefly, cauda epididymis and epididymis were collected in culture dishes in PBS pH 7.4 (0.2 M phosphate, 1.5 M NaCl) containing 5% BSA, 5% non-fat dry milk powder, 1M CaCl<sub>2</sub>, 1M MgCl<sub>2</sub> filtered through a cellulose acetate membrane (Sartorius) and cut into small pieces to release sperm cells. The suspension was loaded into the elutriation chambers, which form part of the centrifuge rotor, using a rotor speed of 3500 revolutions per minute (rpm) and a pump rate of 7ml/min. Mature sperm was eluted by increasing the pump rate to 31ml/min. Purity of the elutriate was confirmed by inspecting the eluted sperm cells under a light microscope.

### **5.6.4. RNA sequencing**

RNA was isolated using a standard Trizol protocol. The quantity and quality of RNAs were determined by Qubit® fluorometer (Life Technologies) and Agilent 2100

Bioanalyser (Agilent Technologies), respectively. Sequencing was done using an Illumina Genome Analyzer (Illumina, San Diego, USA) at Fasteris AG, Geneva, Switzerland. Small RNA libraries were prepared according to a modified Illumina v1.5 protocol. Briefly, small RNAs of <50 nt were purified on an acrylamide gel. Universal miRNA cloning linker (New England Biolabs) instead of 3' adapters and then 5' Illumina adapters were single-stranded ligated with T4 truncated RNA and T4 ligase respectively. The constructs were purified on an acrylamide gel to remove empty adapters then reverse-transcribed and PCR-amplified. The primers used for cDNA synthesis and PCR were designed to insert an index in the 3'- adapter. This index enables assignation of a specific read to the corresponding library, among the multiplexed libraries of one sequencing lane. High-throughput sequencing was performed on a Genome Analyzer HiSeq 2000 for 50 cycles plus 7 cycles to read the indexes. After demultiplexing and adapter removal, an average of 16067416 pass filter reads was obtained in the libraries.

#### **5.6.5. miRNA targets prediction**

The DIANA-microT CDS miRNA target prediction algorithm (225) which is based on potential binding site in the 3' untranslated region of the mRNA and predicted stable thermodynamic binding, was used to predict target genes of miRNAs. Binding score threshold was set to 0.9 (1=highest potential binding predicted, 0=no binding predicted) and only the top 100 targets were considered for each miRNA, to only consider predictions with highest probability.

#### **5.6.6. RNAs injection**

Fertilized oocytes were collected from B6D2F1 (Janvier) females super ovulated by intraperitoneal (ip) injection of 5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotrophin 48 hours later, then mated with B6D2F1 males. One to two picoliters of 0,5 ng/µl solution of total RNA isolated and pooled from sperm from 5 adult MSUS or control males (same samples used for sequencing) dissolved in 0,5 mM Tris-HCl, pH 8.0, 5 uM EDTA were microinjected into the male pronucleus of fertilized eggs using a standard microscope and DNA microinjection method (226).

#### **5.6.7. Behavioral testing**

The experimenter was blind to treatment, and behaviors were monitored by direct observation and videotracking (Viewpoint, France).

##### *Elevated plus maze*

The elevated plus maze consisted of a platform with two open (without walls) and two closed (with walls) arms (dark gray PVC, 30cm x 5cm) elevated 60 cm above the floor. All experiments were performed in red light (15W). Each mouse was placed on the central platform, facing a closed arm, and observed for a 5-min period. The latency to enter an open arm, the time spent in each arm and the total distance moved were automatically recorded by a videotracking system. The number of rearing, protected (body in closed arm) and unprotected (body in opened arm) stretch-attend postures in the center of the maze were manually recorded.

#### *Light/dark box*

Each mouse was placed in the lit compartment (white walls, 130 lux) of a plastic box (40 x 42 x 26cm) split into two unequal compartments (2/3 lit, 1/3 dark compartment with black walls and covered by a black lid) by a divider with an opening (5x5cm). The animal can move freely from the lit to the dark compartment during a 10 mins session. The time spent in each compartment and the latency to enter the dark compartment were measured manually.

#### *Forced swim test*

Mice were placed in a small tank of water (18 cm high, 13 cm diameter,  $18 \pm 1^{\circ}\text{C}$ , filled up to 12 cm) for 5 min. Floating duration was scored manually.

#### **5.6.8. Serum insulin and blood glucose analyses**

For non-fasted baseline measurements of insulin, blood was collected, stored overnight at  $4^{\circ}\text{C}$ , centrifuged for 10 minutes at 2,000 g at  $4^{\circ}\text{C}$ , then serum was collected and stored at  $-80^{\circ}\text{C}$  until analyzed. Insulin was measured in serum using a mouse insulin ELISA (Alpco). The sensitivity of the assay was 0.06 ng/mL, and the intra-assay coefficient of variation was 3.7%. Glucose in non-fasted animals was measured in blood samples at baseline and after acute stress. Mice were restrained for 30 minutes (between 14h00 and 16h00) in a plastic tube and blood samples were collected from a tail nick 0, 15, 30 and 90 minutes after initiation of restraint. For the glucose tolerance test, mice were fasted for 6 hours. Glucose was measured in blood samples at baseline, and 0, 15, 30 and 90 minutes after i.p. injection of 2mg/g body weight glucose in 0.45% saline (injection started at 2 pm). For the insulin tolerance test, mice were fasted for 6 hours. Glucose was measured in blood samples at baseline, and 0, 15, 30, 90 and 120 minutes after i.p. injection of 1mU/g body weight insulin (NovoRapid Novo Nordisk A/S) in sterile 0.9% saline. If blood glucose fell below 1.7 mM/ml, animals were rescued with i.p. injection of 2mg/g glucose and were

removed from the experiment. Glucose level was determined in fresh tail blood using an Accu-Chek Aviva device (Roche).

#### **5.6.9. Caloric intake measurement**

The amount of consumed food was measured for each mouse (4 months old) every 24h. Caloric intake was calculated as the mean amount of food intake over 48 hours in relation to mean body weight (caloric intake = mean food intake/mean body weight).

#### **5.6.10. Cell culture**

Mouse neuroblastoma (N2a) cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum. Approximately 300,000 cells from three different passage number stocks were simultaneously plated in 6-well culture plates. Cells were treated with miScript miRNA mimic (Qiagen) and a negative control siRNA with no known targets in mammalian genome (All Stars Negative siRNA, Qiagen) at 60 nM for 48 hours. Transfections were carried out using lipid-based HiPerfect transfection reagent (Qiagen). Cells were harvested 48 hours after transfection and total RNA was isolated using standardized Trizol protocol.

#### **5.6.11. Western blotting**

Western blotting was performed as previously described (138). Briefly, 30-60 µg proteins were resolved on 10-12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked (Ctnnb1: milk 5%) then incubated in primary and secondary antibodies. Band intensity was determined and quantified using an Odyssey IR scanner (Li-Cor Biosciences). bactin (1:15000; mouse monoclonal; Sigma) was used as internal control. The following antibodies were used: Ctnnb1 (1:2000, mouse monoclonal; BD Biosciences); goat anti-rabbit (IRDye 800nm, 1:10,000; Li-cor Biosciences) and goat anti-mouse (IRDye 680 nm, 1:10,000; Li-Cor Biosciences). Samples from different groups were processed on the same blots. Data are expressed as percent relative to controls.

### **5.6.12. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)**

For miRNAs, DNaseI-treated RNA isolated from pure sperm cells or hippocampal samples (Trizol) was reverse-transcribed (RT) using the miScript reverse transcription kit (Qiagen). RT-qPCR was performed in a LightCycler 480 qPCR (Roche) using miScript probes (Qiagen) according to the manufacturer's recommendations. For normalization of Ct values for miRNAs, we used miR-101b for sperm, ribosomal Rnnu6 for hippocampus and miR-195 for serum. For mRNAs, RT-qPCR were performed using SYBR green (Roche) on a Light-Cycler II 480 (Roche) according to the manufacturer's recommendations. Data for tissue samples were normalized to two endogenous controls (Tubd1 and Hprt), and data for cell samples were normalized to GAPDH. Cycling conditions: 5 min at 95°C, 45 cycles with denaturation (10 sec at 95°C), annealing (10 sec at 60°C) and elongation (10 sec at 72°C). Primers were as follows:

Tubd1	forward:	TCTCTTGCTAACTTGGTGGTCCTC	reverse:
		GCTGGGTCTTAAATCCCTCTACG,	Hprt
		GTTGGGCTTACCTCACTGCTTC	forward:
		reverse: CCTGGTTCATCATCGCTAATCACG,	
Ctnnb1	forward:	ATGGAGCCGGACAGAAAAGC	reverse:
		CTTGCCACTCAGGGAAGGA,	GAPDH forward: CCACTGGTGCTGCCAAGGCT
		reverse: GGCAGGTTCTCCAGGCGGC.	

### **5.6.13. Deep sequencing data analyses**

#### *Overall analysis of small RNAs libraries*

After adapter trimming, sequence reads were sorted based on length (number bp) and only 15-44bp reads were considered for further analysis. The number of reads of each size was counted and normalized to the total number of reads. The obtained counts were averaged across control libraries. Reads were aligned to the mouse genome (UCSC mm9, <http://hgdownload.cse.ucsc.edu/goldenPath/mm9/bigZips/>) (227), ncRNAs and repeat elements from rmsk (UCSC mm9, <http://hgdownload.cse.ucsc.edu/goldenPath/mm9/bigZips/>) and mitochondrial DNA (UCSC mm9, <http://hgdownload.cse.ucsc.edu/goldenPath/mm9/bigZips/>) using a BWA software (228) with mismatch tolerance of up to 2bp for 15-17bp inserts, and 3 and 4bp for 18-38bp and 37-44bp inserts respectively. Sequencing reads were aligned to different selected features (ncRNAs, mtDNA) separately to “force” mapping (sequences of the mouse genome other than the respective feature were masked to prevent alignment to featured regions), and the different features matching each read were determined. The percentage of reads of a given size mapping once or multiple

times (unique or multiple hits) to the mouse genome or to a given feature (100% represents all reads of a given size within a library) was determined and averaged across all control libraries.

#### *Analysis of miRNA and piRNA sequences*

Perfect matches to mature miRNA sequences downloaded from miRBase (229) were identified using custom Perl scripts. One sample was removed from the analysis due to much lower total read count (see Supplementary Figure 3a). Read counts were identified for each miRNA and normalized using DESeq (230). A Wilcox unpaired test on the normalized data was used to identify miRNAs showing a statistically significant difference after MSUS treatment. piRNAs were identified by determining sequences that aligned to annotated piRNA clusters (231) using Bowtie (232). Alignments to piRNA cluster sequences were conducted as a custom-built “genome” with parameters –k 1 –v 0 --best (to select only the best aligning read with 0 mismatch). After inspection to confirm enrichment of piRNA-like sequences (Supplementary Figure 4a and c), all sequences with a length of 26-32 nucleotides and a T as the first nucleotide were selected from the libraries and used for alignment to piRNA clusters. DEseq was then used to normalize read counts for each cluster and the differential expression and statistical significance of the differential expression was calculated using a negative binomial test within the DESeq package.

#### **5.6.14. Statistical analyses**

Two-tailed Student t tests were used to assess statistical significance for behavioral, quantitative RT- qPCR, insulin, bodyweight and caloric intake measurements. The remaining metabolic experiments were analyzed using repeated measurements ANOVAs. All analyzed data matched the requirements for parametric statistical tests (normal distribution). If variance was not homogenous between groups (determined by Levene’s test), adjusted p-value, t-value and degree of freedom were determined. miRNAs were analysed using Mann Whitney U test and piRNAs were analyzed using negative binomial test with and without Bonferoni multiple test correction. Values over two standard deviations away from the mean of each group were considered outliers and excluded from analysis. All statistics were computed with SPSS. Significance was set at  $p < 0.05$  for all tests. Error bars represent SEM in all figures.

## 6. Discussion

### 6.1. Significance

In this thesis we describe for the first time the effect of early life stress on the offspring in a context-dependent manner. Further, we propose a treatment to counteract such transmission, and provide a mechanism that contributes to the inheritance of the phenotype.

#### 6.1.1. Effects of early life stress

Prior studies, including work from our own laboratory, focused primarily on the maladaptive changes triggered by chronic stress paradigms (22, 49, 50, 233). Stress might however also elicited advantageous effects (151). The effects depend on the quality and quantity of stressor (151, 234). Further, the same stress manipulation might yield different results depending on the environment it is tested in (54). Different hypotheses have been put forward to explain the complex and often contradictory effects observed in response to early life adversity. The match/mismatch (MM) model (55) hypothesizes that adverse early life events constitute a potential source of adaptation, preparing the exposed individuals to better cope with similar environmental conditions later in life. The “for-better-and-for-worse” (71) model postulates that genetic risk factors should be viewed only in relation to context: vulnerable individuals of a specific genotype in one environment may actually turn out resilient in another environment. Therefore, specific single nucleotide polymorphisms that have been reported to constitute “risk” factors for the development of certain diseases should be reconsidered as “susceptibility” factors. Susceptibility, in this context does not refer to a malleable disease predisposition, it rather describes a state of high responsiveness, disregard a qualitative weighting. Contrarily, the cumulative stress model (235) proposes that accumulating exposure to stressors will increase disease risk. This model might apply more to very severe stress, opposing to a moderately challenging environment that promotes active coping. Together the three concepts were recently reconciled and channeled into the three hit model (5), which elegantly summarizes the current way of thinking in the field. In this model, the first hit is represented by genetic variation and the second and third hit by specific environmental conditions. Thus gene (hit1) x early life environment (hit-2) interactions prime a developing organism to a susceptible state or phenotype. This process is likely mediated by epigenetic mechanisms (57, 118). A susceptible state/phenotype then exposed to a specific context (hit3a) later in life might lead to vulnerability (increased disease predisposition), or when exposed to a different environment

(hit3b) alternatively lead to resilience (increased fitness) (5). The outcome might be due to a match or mismatch of exposures, but can also result from an accumulation in cases of very severe environmental exposures.

In terms of the MSUS model the three hit model is only partially applicable, since the hits spread across generations. In the first generation of MSUS animals the first hit, genetic susceptibility, is not eligible, since we use an inbred mouse strain, which is genetically identical. Thus there are only two hits in the first generation. One hit, early life environment, is represented by the early life stress in F1 animals and the other hit consists of the environment at the time of testing. There can be a match or a mismatch, hence potentially leading to improved or impaired performance. Since we know that epigenetic modifications can be inherited, I would like to stretch the concept of the first hit to epigenetic variation. The second generation's first hit is thus a susceptible epigenome, primed by the second hit of the previous generation. The second hit could be considered as a lack of early life adversity. Then, the third hit again would consist of the environment at the time of testing. These three hits again can yield different performances depending on how much mismatch there is.

On a molecular level we found that the environmental hits in the first generations alter mineralocorticoid receptor (MR) expression across generations and confer a specific epigenetic signature to this gene in the sperm and in the brain of the offspring. The epigenetic profile of the MR gene in sperm thus constitutes the first hit, the epigenetic susceptibility of the F2 generation. Our data suggest that this epigenetic susceptibility state has functional relevance for the behavioral observations during the third hit, represented by the environment at the time of testing in the second generation. These results broaden the knowledge on epigenetic regulation of stress receptors, such as the Glucocorticoidreceptor, previously conceptually integrated in stress models (11, 55) to the epigenetic regulation of the mine.

By providing both maladaptive and beneficial observations our study clearly demonstrates that the terms "beneficial" and "maladaptive" are only to be used in relation with a specific environmental context and (epi)genetic predisposition. Thus our findings complement the three-hit model, by expanding this concept to a transgenerational priming- concept.

### **6.1.2. Context dependency of acquired traits**

It is important to note that not all epigenetically inherited traits are context-dependent. Traits induced by endocrine disruptors that significantly increase cancer predisposition

or infertility (19, 39, 43, 236, 237) will clearly be maladaptive in any circumstances. In comparison, the MSUS exposure constitutes a rather mild manipulation with subtle outcome. Despite leading to epigenetic inheritance of the acquired traits, the induced epigenetic alterations are not as stable as mutations, they may again be modified, or accumulate and interact with other alterations. If encountering a mismatching environment, certain epigenetic marks might trigger detrimental responses that would lead to negative natural selection. Hence, when putting epigenetic inheritance of acquired traits in an evolutionary context one has to distinguish between the aforementioned certainly maladaptive traits and other more subtle changes such as those observed in the MSUS model.

#### **6.1.3. Contingency in the inheritance of acquired traits**

The question then arises whether the propagation of epigenetically primed traits is directed or stochastic. If the propagation was stochastic the disadvantageous traits would be considered a negative byproduct that has to be tolerated for a species to profit from the inheritance of other more advantageous traits. An advantageous trait could indeed be an increased susceptibility to changes or increased responsiveness. An increased responsiveness does not seem intuitively directed towards disadvantageous adaption or advantageous adaption. But despite not being channeled in a specific direction it directs the susceptibility towards a specific gene (on a molecular level) or a specific trait (on a behavioral level). Evidence for maladaptive traits being a mere side product comes from a study on invading transposons in *Arabidopsis Thaliana*. This study shows that gene expression is negatively correlated with the density of methylated transposons. Natural selection is detectable for methylated transposons near genes but not for unmethylated transposons or for transposons far from genes. Evolutionary older transposons are further away from genes. These findings suggest that silencing of invading transposons can spread to neighboring genes. If these genes are vital to the organism they would immediately lead to negative selection (238). If the genes are not essential they would get silenced by DNAmE to avoid activation of the neighboring transposon. This would then show as a decreased gene expression, which if extrapolated to animals might lead to inappropriate behavioral adaptations.

On the contrary, stochastic epigenetic evolution would merely lead to an increase in variability in response to environmental fluctuations. This would result in a more diverse offspring population, bearing both, changes that confer increased responsiveness/susceptibility to genes or traits and changes that are clearly

maladaptive independent of the context. By chance, some adaptations may be ideally suited to fit the environment encountered by the organism, provide an advantage and thus propagate. A recent study provides evidence that certain epigenetic states make regions of the genome prone to mutagenesis (239). It shows that spots of high mutability, associated with diseases such as schizophrenia or bipolar disorder, strongly correlate with regions prone to non-allelic homologous recombination mediated by low copy number repeats and hypomethylated regions (239). This means that those traits that enhance fitness could potentially even get engraved in the genome genetically, thereby becoming stable genetic marks.

#### **6.1.4. Maintenance of flexibility of epigenetic signatures**

However, if epigenetic alterations are not turned into genetic information, they seem to remain flexible and reversible (78). This is supported by our demonstration that EE can reverse some of the detrimental transgenerational effects induced by MSUS (35). These results reinforce previous findings on the effect of environmental enrichment on the subsequent generation. This study showed a rescue of a genetically induced impairment of long-term potentiation. The rescue was perpetuated to the offspring. Our findings broaden the concept of an inheritable rescue by showing that environmental enrichment can also rescue epigenetically primed deficits across generations. Similar findings were obtained by a study in rats (36). A rescue of pregestational stress induced behavioral deficits in the offspring was achieved by exposing the dam to an enriched environment prior gestation. Since the transmission occurred through females it is not possible to exclude a potential behavioral transfer to the offspring by altered maternal care. Our study is thus the first to show transgenerational rescue of stress induced behaviors through the male line.

#### **6.1.5. Small RNAs as carriers of information across generations**

We attribute the heritability of early life stress induced traits to small sperm RNAs. A similar mechanism has been proposed by various studies on epigenetic inheritance in *Drosophila* (45) and *C. elegans* (44, 46, 100, 101, 203, 204, 240). Our study is the first to report that these RNAs are critically involved in epigenetic inheritance of environmentally triggered traits in mammals, thus providing further proof for their functional relevance. Previous transgenerational studies on the impact of chronic stress or drug-exposure in mammals have only focused on DNA-methylation patterns or histone acetylation at single genes after chronic stress or drug exposure (22, 41).

However, the likelihood that such complex manipulations affect only single genes is very low. Small RNAs, in contrast, have a variety of gene targets and thereby can impact gene regulation on a genome wide level. Nevertheless, small RNAs most probably constitute only an intermediate epigenetic information carrier. The information may later be consolidated by directing DNA methylation and histone post translational modifications (92, 241). This hypothesis is supported by the fact that we failed to detect altered candidate miRNAs in the sperm of F2 animals, while the behavioral phenotype persists in following generations.

## 6.2. Limitations

### 6.2.1. Specific function of altered non-coding sperm RNAs

Despite providing evidence that sperm RNAs contribute to the transgenerational transmission of the effect of early life stress, we have not yet determined a specific time point when these sperm RNAs carry out their function. Our experimental design that uses RNA injection into fertilized oocytes and investigates the impact on the generated offspring provides evidence that some sperm RNAs are functionally relevant in the zygote. We were also able to show that one sperm miRNA is upregulated in the zygote, suggesting delivery from sperm to the oocyte in natural mating conditions. Therefore, we believe that this serves a purpose. Some RNAs might exert their function shortly after fertilization (94, 107, 111) by affecting zygotic erasure of epigenetic marks (242). Another interesting possibility is that the delivered RNAs might enter a self-amplification cycle. Once amplified, they might impact the reinstatement of epigenetic marks after zygotic reprogramming (105, 109). It is also likely that sperm RNAs are additionally crucial during sperm maturation. This hypothesis is supported by the finding that a proportion of sperm RNAs associate with the nuclear matrix (243), where they are believed to impact chromatin remodeling. Furthermore, mature sperm miRNAs mainly align to promoter regions (207). This raises the speculation that they could bind to paternal DNA and thereby prevent histone to protamine transition during sperm nuclear remodeling. Nuclear remodeling spares f.i. the imprinted gene IGF-2 (119). The resulting retained histones can thus prime this region for early activity. It is likely, that many other regions of the nuclear matrix are influenced in the same way by RNA, which would hence indirectly contribute to transgenerational inheritance (156).

#### **6.2.2. Induction of epigenetic alterations**

Another major unanswered question concerns the induction of the epigenetic alteration through MSUS. It is known that miRNAs can be taken up by the blood and be delivered to other tissues (51). MSUS mice show alteration of miRNA expression in several tissues (including serum), it is however not feasible to date to depict the origin of these miRNAs. They might all stem from the same tissue, or all the analyzed tissues might respond to stress in a similar manner. Hence we do not know whether the altered sperm RNAs are produced in sperm or whether they originate elsewhere and are taken up by sperm. Further, we are not able to identify the initial chemical trigger in MSUS mice although we do provide some evidence that intraperitoneal injection of corticosterone also increases the expression of miR-375 in the hippocampus.

#### **6.2.3. Other potential carriers of information across generations**

Certainly, looking at one epigenetic mechanism alone will not be able to grasp the full complexity of alterations induced by early life stress are transmitted. We tried to correlate our sperm RNA sequencing data with sperm DNA MeDIP data, but did not find any correlations. We do, however, suspect that this might be due to a low sensitivity of the DNA methylation technique, since we were not able to verify the MeDIP data by follow up single-gene pyrosequencing.

#### **6.2.4. Mechanisms underlying the transmission of behavioral flexibility**

Ideally we could have also linked the study of the impact of early life stress on behavioral flexibility to the mechanistic involvement of RNAs. However, the RNA injections are very tedious and produced only a limited number of offspring. Therefore we decided to focus on a broad phenotyping strategy of the animals obtained, rather than using them for one long-term experiment assessing behavioral-flexibility. It is questionable whether the offspring of MSUS RNA injected animals would also show similar facilitations in goal directed behavior and reversal learning as the natural offspring of MSUS males. We showed that the molecular mechanism critically involved in this phenotype depends on histone PTMs at the mineralocorticoid receptor gene. The mechanism for the transgenerational impact on epigenetic regulation of the mineralocorticoid receptor remains to be determined, although we show altered DNA methylation in sperm of the promoter of the gene. Recent findings suggest that the retention of altered histone post translational modifications in sperm could account for

such effects, but so far no laboratory managed to retrieve enough chromatin from mature sperm cells to conduct quantitative, group-wise analysis on single histone post translational modifications in mouse sperm.

#### **6.2.5. Mechanisms underlying the reversal of MSUS induced traits**

Regarding the study of the potential of environmental enrichment to reverse the phenotype induced by MSUS, it is evident that this manipulation most likely does not lead to a specific molecular reversal. This becomes apparent since not all behavioral alterations induced by MSUS are reversed and the enrichment- induced alterations do not have a classical reversal pattern. Rather, exposure to an enriched environment seems to exert some additional effects that are also transmitted to the next generation but do mask some MSUS-induced alterations. Most likely, distinct mechanisms are employed by this exposure. Hence, a precise study of the molecular mechanisms underlying the effects of enriched environment is needed to conclusively interpret the findings on the behavioral reversal, judge their therapeutic potential and put them into an evolutionary context.

### **6.3. Outlook**

Follow-up experiments should try to elucidate in greater detail the molecular alterations in the brain and sperm of MSUS F1 and F2 animals. A careful study of their potential reversal through exposure to an enriched environment should be conducted. Additionally, the investigation could focus on molecular alterations in brain and sperm of animals exposed only to environmental enrichment and compare them to control animals. Such analysis could detect confounding variables that mask an explicit reversal of MSUS induced behavioral traits by environmental enrichment.

To continue the study on a potential mechanism for the transgenerational effect of early life stress on the epigenetic regulation of the mineralocorticoid receptor, future experiments will require more refined techniques to precipitate chromatin from a single histone post-translational modification in sperm. A detailed analysis of the histone marks that were altered in the brain of F2 MSUS females, in sperm at the mineralocorticoid receptor gene would then become feasible.

The study of the implication of sperm RNAs in the transmission of early life stress induced traits should further focus on other classes of sperm RNAs. These RNAs

include long-noncoding RNAs and mRNAs. Putting such data into the context of genome-wide assessment of other epigenetic marks in sperm, such as DNA methylation and histone post translational modifications, will help substantially to bring this emerging field forward.

## 7. References

1. E. R. de Kloet, M. Joels, F. Holsboer, Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* **6**, 463 (2005).
2. B. S. McEwen, Understanding the potency of stressful early life experiences on brain and body function. *Metabolism* **57**, S11 (2008).
3. T. L. Bale, Stress sensitivity and the development of affective disorders. *Hormones and behavior* **50**, 529 (Nov, 2006).
4. E. R. de Kloet, Hormones, brain and stress. *Endocr Regul* **37**, 51 (Jun, 2003).
5. N. P. Daskalakis, R. C. Bagot, K. J. Parker, C. H. Vinkers, E. R. de Kloet, The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinology*, (Jul 6, 2013).
6. I. C. Weaver, Epigenetic programming by maternal behavior and pharmacological intervention. Nature versus nurture: let's call the whole thing off. *Epigenetics* **2**, 22 (Jan-Mar, 2007).
7. T. Kouzarides, Chromatin modifications and their function. *Cell* **128**, 693 (Feb 23, 2007).
8. R. Jaenisch, A. Bird, Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* **33 Suppl**, 245 (Mar, 2003).
9. P. A. Jones, D. Takai, The role of DNA methylation in mammalian epigenetics. *Science* **293**, 1068 (Aug 10, 2001).
10. R. L. Jirtle, M. K. Skinner, Environmental epigenomics and disease susceptibility. *Nat Rev Genet* **8**, 253 (Apr, 2007).
11. I. C. Weaver *et al.*, Epigenetic programming by maternal behavior. *Nat Neurosci* **7**, 847 (Aug, 2004).
12. N. M. Tsankova *et al.*, Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* **9**, 519 (2006).
13. I. B. Zovkic, M. C. Guzman-Karlsson, J. D. Sweatt, Epigenetic regulation of memory formation and maintenance. *Learn Mem* **20**, 61 (2013).
14. E. J. Nestler, Epigenetic mechanisms of drug addiction. *Neuropharmacology*, (Apr 30, 2013).
15. R. G. Hunter, B. S. McEwen, Stress and anxiety across the lifespan: structural plasticity and epigenetic regulation. *Epigenomics* **5**, 177 (Apr, 2013).
16. J. J. Day *et al.*, DNA methylation regulates associative reward learning. *Nat Neurosci*, (Aug 25, 2013).
17. C. A. Miller, J. D. Sweatt, Covalent modification of DNA regulates memory formation. *Neuron* **53**, 857 (Mar 15, 2007).
18. J. D. Sweatt, Experience-dependent epigenetic modifications in the central nervous system. *Biol Psychiatry* **65**, 191 (Feb 1, 2009).
19. C. Guerrero-Bosagna, M. Settles, B. Lucker, M. K. Skinner, Epigenetic Transgenerational Actions of Vinclozolin on Promoter Regions of the Sperm Epigenome. *PLoS one* **5**, e13100 (2010).
20. C. Stouder, A. Paoloni-Giacobino, Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. *Reproduction* **139**, 373 (February 1, 2010, 2010).
21. M. K. Skinner, C. G. Haque, E. Nilsson, R. Bhandari, J. R. McCarrey, Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. *PLoS one* **8**, e66318 (2013).
22. T. B. Franklin *et al.*, Epigenetic transmission of the impact of early stress across generations. *Biological psychiatry* **68**, 408 (Sep 1, 2010).
23. A. Soubry *et al.*, Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort. *BMC medicine* **11**, 29 (2013).
24. E. J. Radford *et al.*, An unbiased assessment of the role of imprinted genes in an intergenerational model of developmental programming. *PLoS Genet* **8**, e1002605 (2012).
25. F. M. Vassoler, S. L. White, H. D. Schmidt, G. Sadri-Vakili, R. C. Pierce, Epigenetic inheritance of a cocaine-resistance phenotype. *Nature neuroscience* **16**, 42 (Jan, 2013).

26. D. M. Dietz *et al.*, Paternal Transmission of Stress-Induced Pathologies. *Biological Psychiatry* **70**, 408 (2011).
27. C. P. Morgan, T. L. Bale, Early Prenatal Stress Epigenetically Programs Dysmasculinization in Second-Generation Offspring via the Paternal Lineage. *The Journal of Neuroscience* **31**, 11748 (August 17, 2011, 2011).
28. A. B. Rodgers, C. P. Morgan, S. L. Bronson, S. Revello, T. L. Bale, Paternal Stress Exposure Alters Sperm MicroRNA Content and Reprograms Offspring HPA Stress Axis Regulation. *J Neurosci* **33**, 9003 (May 22, 2013).
29. T. L. Roth, F. D. Lubin, A. J. Funk, J. D. Sweatt, Lasting Epigenetic Influence of Early-Life Adversity on the BDNF Gene. *Biological Psychiatry* **65**, 760 (2009).
30. D. C. Benyshek, C. S. Johnston, J. F. Martin, Glucose metabolism is altered in the adequately-nourished grand-offspring (F3 generation) of rats malnourished during gestation and perinatal life. *Diabetologia* **49**, 1117 (May, 2006).
31. J. C. Jimenez-Chillaron *et al.*, Beta-cell secretory dysfunction in the pathogenesis of low birth weight-associated diabetes: a murine model. *Diabetes* **54**, 702 (Mar, 2005).
32. J. C. Jimenez-Chillaron *et al.*, Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice. *Diabetes* **58**, 460 (Feb, 2009).
33. B. T. Heijmans *et al.*, Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* **105**, 17046 (Nov 4, 2008).
34. M. E. Pembrey *et al.*, Sex-specific, male-line transgenerational responses in humans. *European journal of human genetics : EJHG* **14**, 159 (Feb, 2006).
35. J. A. Arai, S. Li, D. M. Hartley, L. A. Feig, Transgenerational rescue of a genetic defect in long-term potentiation and memory formation by juvenile enrichment. *J Neurosci* **29**, 1496 (Feb 4, 2009).
36. M. Leshem, J. Schulkin, Transgenerational effects of infantile adversity and enrichment in male and female rats. *Dev Psychobiol* **54**, 169 (Mar, 2012).
37. A. A. Agrawal, C. Laforsch, R. Tollrian, Transgenerational induction of defences in animals and plants. *Nature* **401**, 60 (1999).
38. L. V. Harper, Epigenetic inheritance and the intergenerational transfer of experience. *Psychol Bull* **131**, 340 (May, 2005).
39. M. D. Anway, A. S. Cupp, M. Uzumcu, M. K. Skinner, Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility. *Science* **308**, 1466 (June 3, 2005, 2005).
40. M. K. Skinner, M. D. Anway, M. I. Savenkova, A. C. Gore, D. Crews, Transgenerational Epigenetic Programming of the Brain Transcriptome and Anxiety Behavior. *PloS one* **3**, e3745 (2008).
41. D. Crews *et al.*, Epigenetic transgenerational inheritance of altered stress responses. *Proceedings of the National Academy of Sciences* **109**, 9143 (June 5, 2012, 2012).
42. J. T. Wolstenholme *et al.*, Gestational Exposure to Bisphenol A Produces Transgenerational Changes in Behaviors and Gene Expression. *Endocrinology* **153**, 3828 (June 15, 2012, 2012).
43. C. Guerrero-Bosagna *et al.*, Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reprod Toxicol* **34**, 694 (Dec, 2012).
44. A. Ashe *et al.*, piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88 (Jul 6, 2012).
45. T. Grentzinger *et al.*, piRNA-mediated transgenerational inheritance of an acquired trait. *Genome research* **22**, 1877 (Oct, 2012).
46. M. Shirayama *et al.*, piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* **150**, 65 (Jul 6, 2012).
47. K. S. Kendler, Twin studies of psychiatric illness: an update. *Arch Gen Psychiatry* **58**, 1005 (Nov, 2001).
48. J. Bohacek, K. Gapp, B. J. Saab, I. M. Mansuy, Transgenerational epigenetic effects on brain functions. *Biol Psychiatry* **73**, 313 (Feb 15, 2013).
49. T. B. Franklin, N. Linder, H. Russig, B. Thöny, I. M. Mansuy, Influence of Early Stress on Social Abilities and Serotonergic Functions across Generations in Mice. *PloS one* **6**, e21842 (2011).
50. I. C. Weiss, T. B. Franklin, S. Vizi, I. M. Mansuy, Inheritable effect of unpredictable maternal separation on behavioral responses in mice. *Frontiers in Behavioral Neuroscience* **5**, doi: 10.3389/fnbeh.2011.00003 (2011-February-4, 2011).

51. X. Chen, H. Liang, J. Zhang, K. Zen, C. Y. Zhang, Secreted microRNAs: a new form of intercellular communication. *Trends in cell biology* **22**, 125 (Mar, 2012).
52. B. R. B. D. T. Smith, Fitness consequences of personality: a meta-analysis. *Behavioral Ecology*, (22. January 2008, 2008).
53. F. Mery, Natural variation in learning and memory. *Current opinion in neurobiology* **23**, 52 (Feb, 2013).
54. N. P. Daskalakis, M. S. Oitzl, H. Schachinger, D. L. Champagne, E. R. de Kloet, Testing the cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life adversity. *Physiology & behavior* **106**, 707 (Jul 16, 2012).
55. E. R. de Kloet, M. Joels, F. Holsboer, Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* **6**, 463 (Jun, 2005).
56. N. P. Daskalakis, R. C. Bagot, K. J. Parker, C. H. Vinkers, E. R. de Kloet, The three-hit concept of vulnerability and resilience: Toward understanding adaptation to early-life adversity outcome. *Psychoneuroendocrinology* **38**, 1858 (Sep, 2013).
57. T. Klengel *et al.*, Allele-specific FKBP5 DNA demethylation mediates gene-childhood trauma interactions. *Nat Neurosci* **16**, 33 (Jan, 2013).
58. S. Uchida *et al.*, Epigenetic status of Gdnf in the ventral striatum determines susceptibility and adaptation to daily stressful events. *Neuron* **69**, 359 (Jan 27, 2011).
59. K. Luger, A. W. Mader, R. K. Richmond, D. F. Sargent, T. J. Richmond, Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 251 (Sep 18, 1997).
60. J. Tost, DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Methods Mol Biol* **507**, 3 (2009).
61. R. Y. Tweedie-Cullen, J. M. Reck, I. M. Mansuy, Comprehensive Mapping of Post-Translational Modifications on Synaptic, Nuclear, and Histone Proteins in the Adult Mouse Brain. *Journal of Proteome Research* **8**, 4966 (2009/11/06, 2009).
62. M. Ghildiyal, P. D. Zamore, Small silencing RNAs: an expanding universe. *Nat Rev Genet* **10**, 94 (Feb, 2009).
63. F. Santos, A. H. Peters, A. P. Otte, W. Reik, W. Dean, Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. *Developmental biology* **280**, 225 (Apr 1, 2005).
64. E. Nederhof, M. V. Schmidt, Mismatch or cumulative stress: toward an integrated hypothesis of programming effects. *Physiol Behav* **106**, 691 (Jul 16, 2012).
65. R. Lister *et al.*, Global epigenomic reconfiguration during mammalian brain development. *Science* **341**, 1237905 (Aug 9, 2013).
66. N. M. Tsankova *et al.*, Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* **9**, 519 (Apr, 2006).
67. J. Tan *et al.*, Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev* **21**, 1050 (May 1, 2007).
68. J. Borgel *et al.*, Targets and dynamics of promoter DNA methylation during early mouse development. *Nat Genet* **42**, 1093 (2010).
69. K. Gapp, B. T. Woldemichael, J. Bohacek, I. M. Mansuy, Epigenetic regulation in neurodevelopment and neurodegenerative diseases. *Neuroscience*, (Dec 19, 2012).
70. O. Kofman, The role of prenatal stress in the etiology of developmental behavioural disorders. *Neuroscience and biobehavioral reviews* **26**, 457 (Jun, 2002).
71. J. Belsky, K. M. Beaver, Cumulative-genetic plasticity, parenting and adolescent self-regulation. *Journal of child psychology and psychiatry, and allied disciplines* **52**, 619 (May, 2011).
72. L. P. Spear, Heightened stress responsivity and emotional reactivity during pubertal maturation: Implications for psychopathology. *Development and psychopathology* **21**, 87 (Winter, 2009).
73. P. F. Sullivan, M. C. Neale, K. S. Kendler, Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatry* **157**, 1552 (Oct, 2000).
74. H. K. Kim, D. M. Capaldi, K. C. Pears, D. C. Kerr, L. D. Owen, Intergenerational transmission of internalising and externalising behaviours across three generations: gender-specific pathways. *Crim Behav Ment Health* **19**, 125 (2009).
75. A. S. Khashan *et al.*, Higher risk of offspring schizophrenia following antenatal maternal exposure to severe adverse life events. *Arch Gen Psychiatry* **65**, 146 (Feb, 2008).

76. D. K. Kinney, A. M. Miller, D. J. Crowley, E. Huang, E. Gerber, Autism prevalence following prenatal exposure to hurricanes and tropical storms in Louisiana. *Journal of autism and developmental disorders* **38**, 481 (Mar, 2008).
77. J. van Os, J. P. Seltin, Prenatal exposure to maternal stress and subsequent schizophrenia. The May 1940 invasion of The Netherlands. *The British journal of psychiatry : the journal of mental science* **172**, 324 (Apr, 1998).
78. I. C. Weaver *et al.*, Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *J Neurosci* **25**, 11045 (Nov 23, 2005).
79. P. D. Gluckman, M. A. Hanson, T. Buklijas, F. M. Low, A. S. Beedle, Epigenetic mechanisms that underpin metabolic and cardiovascular diseases. *Nature reviews. Endocrinology* **5**, 401 (Jul, 2009).
80. D. A. Chu, L. M. Williams, A. W. Harris, R. A. Bryant, J. M. Gatt, Early life trauma predicts self-reported levels of depressive and anxiety symptoms in nonclinical community adults: Relative contributions of early life stressor types and adult trauma exposure. *Journal of psychiatric research* **47**, 23 (Jan, 2013).
81. C. A. Smith, T. O. Ireland, T. P. Thornberry, Adolescent maltreatment and its impact on young adult antisocial behavior. *Child abuse & neglect* **29**, 1099 (Oct, 2005).
82. C. Marquez *et al.*, Peripuberty stress leads to abnormal aggression, altered amygdala and orbitofrontal reactivity and increased prefrontal MAOA gene expression. *Translational psychiatry* **3**, e216 (2013).
83. V. Veenit, M. I. Cordero, S. Tzanoulinou, C. Sandi, Increased corticosterone in peripubertal rats leads to long-lasting alterations in social exploration and aggression. *Front Behav Neurosci* **7**, 26 (2013).
84. M. A. Enoch, The influence of gene-environment interactions on the development of alcoholism and drug dependence. *Current psychiatry reports* **14**, 150 (Apr, 2012).
85. S. Macri, G. J. Mason, H. Wurbel, Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. *The European journal of neuroscience* **20**, 1017 (Aug, 2004).
86. T. Z. Baram *et al.*, Fragmentation and unpredictability of early-life experience in mental disorders. *Am J Psychiatry* **169**, 907 (Sep, 2012).
87. J. Nithianantharajah, A. J. Hannan, Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nat Rev Neurosci* **7**, 697 (Sep, 2006).
88. C. Dalla, P. M. Pitychoutis, N. Kokras, Z. Papadopoulou-Daifoti, Sex differences in response to stress and expression of depressive-like behaviours in the rat. *Current topics in behavioral neurosciences* **8**, 97 (2011).
89. D. Peleg-Raibstein, E. Luca, C. Wolfrum, Maternal high-fat diet in mice programs emotional behavior in adulthood. *Behav Brain Res* **233**, 398 (Aug 1, 2012).
90. T. Buchenauer *et al.*, Diet-induced obesity alters behavior as well as serum levels of corticosterone in F344 rats. *Physiology & behavior* **98**, 563 (Dec 7, 2009).
91. A. Prasad, C. Prasad, Short-term consumption of a diet rich in fat decreases anxiety response in adult male rats. *Physiology & behavior* **60**, 1039 (Sep, 1996).
92. A. Kanhere *et al.*, Short RNAs are transcribed from repressed polycomb target genes and interact with polycomb repressive complex-2. *Mol Cell* **38**, 675 (Jun 11, 2010).
93. A. L. Roberts *et al.*, Posttraumatic stress disorder across two generations: concordance and mechanisms in a population-based sample. *Biol Psychiatry* **72**, 505 (Sep 15, 2012).
94. K. D. Wagner *et al.*, RNA induction and inheritance of epigenetic cardiac hypertrophy in the mouse. *Dev Cell* **14**, 962 (Jun, 2008).
95. E. E. Eichler *et al.*, Missing heritability and strategies for finding the underlying causes of complex disease. *Nat Rev Genet* **11**, 446 (2010).
96. M. J. Millan *et al.*, Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nat Rev Drug Discov* **11**, 141 (2012).
97. T. B. Franklin, I. M. Mansuy, Epigenetic inheritance in mammals: Evidence for the impact of adverse environmental effects. *Neurobiology of Disease* **39**, 61 (2010).
98. P. O. McGowan *et al.*, Broad epigenetic signature of maternal care in the brain of adult rats. *PloS one* **6**, e14739 (2011).
99. M. I. Cordero *et al.*, Evidence for biological roots in the transgenerational transmission of intimate partner violence. *Translational psychiatry* **2**, e106 (2012).

100. B. A. Buckley *et al.*, A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* **489**, 447 (Sep 20, 2012).
101. O. Rechavi, G. Minevich, O. Hobert, Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. *Cell* **147**, 1248 (Dec 9, 2011).
102. M. K. Skinner, What is an epigenetic transgenerational phenotype? F3 or F2. *Reprod Toxicol* **25**, 2 (Jan, 2008).
103. U. Grossniklaus, B. Kelly, A. C. Ferguson-Smith, M. Pembrey, S. Lindquist, Transgenerational epigenetic inheritance: how important is it? *Nat Rev Genet* **14**, 228 (Mar, 2013).
104. Y. Li, C. Lalancette, D. Miller, S. A. Krawetz, Characterization of nucleohistone and nucleoprotamine components in the mature human sperm nucleus. *Asian journal of andrology* **10**, 535 (Jul, 2008).
105. A. C. Ravelli *et al.*, Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351**, 173 (Jan 17, 1998).
106. P. Hajkova *et al.*, Chromatin dynamics during epigenetic reprogramming in the mouse germ line. *Nature* **452**, 877 (Apr 17, 2008).
107. V. Grandjean *et al.*, The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth. *Development* **136**, 3647 (Nov, 2009).
108. J. Oswald *et al.*, Active demethylation of the paternal genome in the mouse zygote. *Current biology : CB* **10**, 475 (Apr 20, 2000).
109. A. L. Fowden, C. Sibley, W. Reik, M. Constancia, Imprinted genes, placental development and fetal growth. *Hormone research* **65 Suppl 3**, 50 (2006).
110. J. Castillo, L. Simon, S. de Mateo, S. Lewis, R. Oliva, Protamine/DNA ratios and DNA damage in native and density gradient centrifuged sperm from infertile patients. *Journal of andrology* **32**, 324 (May-Jun, 2011).
111. M. Rassoulzadegan *et al.*, RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* **441**, 469 (2006).
112. Y. Seki *et al.*, Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Developmental biology* **278**, 440 (Feb 15, 2005).
113. J. A. Hackett *et al.*, Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. *Science* **339**, 448 (Jan 25, 2013).
114. D. Bourc'his, G. L. Xu, C. S. Lin, B. Bollman, T. H. Bestor, Dnmt3L and the establishment of maternal genomic imprints. *Science* **294**, 2536 (Dec 21, 2001).
115. T. L. Davis, J. M. Trasler, S. B. Moss, G. J. Yang, M. S. Bartolomei, Acquisition of the H19 methylation imprint occurs differentially on the parental alleles during spermatogenesis. *Genomics* **58**, 18 (May 15, 1999).
116. E. Li, C. Beard, R. Jaenisch, Role for DNA methylation in genomic imprinting. *Nature* **366**, 362 (Nov 25, 1993).
117. U. Brykcynska *et al.*, Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol* **17**, 679 (2010).
118. H. E. Covington, 3rd *et al.*, A role for repressive histone methylation in cocaine-induced vulnerability to stress. *Neuron* **71**, 656 (Aug 25, 2011).
119. S. M. Wykes, S. A. Krawetz, The structural organization of sperm chromatin. *The Journal of biological chemistry* **278**, 29471 (Aug 8, 2003).
120. E. W. Tobi *et al.*, Prenatal famine and genetic variation are independently and additively associated with DNA methylation at regulatory loci within IGF2/H19. *PloS one* **7**, e37933 (2012).
121. E. W. Tobi *et al.*, DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Human molecular genetics* **18**, 4046 (Nov 1, 2009).
122. E. Susser *et al.*, Schizophrenia after prenatal famine. Further evidence. *Arch Gen Psychiatry* **53**, 25 (Jan, 1996).
123. H. Sasaki, Y. Matsui, Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nat Rev Genet* **9**, 129 (Feb, 2008).
124. M. Wan *et al.*, Inducible mouse models illuminate parameters influencing epigenetic inheritance. *Development* **140**, 843 (Feb, 2013).
125. L. Angelucci *et al.*, A model for later-life effects of perinatal drug exposure: maternal hormone mediation. *Neurobehavioral toxicology and teratology* **7**, 511 (Sep-Oct, 1985).

126. P. O. McGowan, M. Szyf, The epigenetics of social adversity in early life: implications for mental health outcomes. *Neurobiology of disease* **39**, 66 (Jul, 2010).
127. J. Bohacek, I. M. Mansuy, Epigenetic inheritance of disease and disease risk. *Neuropsychopharmacology* **38**, 220 (Jan, 2013).
128. C. Heim, E. B. Binder, Current research trends in early life stress and depression: review of human studies on sensitive periods, gene-environment interactions, and epigenetics. *Experimental neurology* **233**, 102 (Jan, 2012).
129. E. McCrory, S. A. De Brito, E. Viding, Research review: the neurobiology and genetics of maltreatment and adversity. *Journal of child psychology and psychiatry, and allied disciplines* **51**, 1079 (Oct, 2010).
130. W. T. Boyce, B. J. Ellis, Biological sensitivity to context: I. An evolutionary-developmental theory of the origins and functions of stress reactivity. *Dev Psychopathol* **17**, 271 (Spring, 2005).
131. E. Chen, G. E. Miller, "Shift-and-Persist" Strategies: Why Being Low in Socioeconomic Status isn't Always Bad for Health. *Perspectives on psychological science : a journal of the Association for Psychological Science* **7**, 135 (Mar 1, 2012).
132. A. Sih, Effects of early stress on behavioral syndromes: an integrated adaptive perspective. *Neurosci Biobehav Rev* **35**, 1452 (Jun, 2011).
133. P. N. Cheifetz, The daily rhythm of the secretion of corticotrophin and corticosterone in rats and mice. *J Endocrinol* **49**, xi (Mar, 1971).
134. S. Koot, W. Adriani, L. Sas, R. van den Bos, G. Laviola, Home cage testing of delay discounting in rats. *Behav Res Methods* **41**, 1169 (Nov, 2009).
135. T. Endo *et al.*, Automated test of behavioral flexibility in mice using a behavioral sequencing task in IntelliCage. *Behav Brain Res* **221**, 172 (Aug 1, 2011).
136. E. M. Bowers *et al.*, Virtual ligand screening of the p300/CBP histone acetyltransferase: identification of a selective small molecule inhibitor. *Chemistry & biology* **17**, 471 (May 28, 2010).
137. T. C. Wu *et al.*, Mineralocorticoid receptor antagonist spironolactone prevents chronic corticosterone induced depression-like behavior. *Psychoneuroendocrinology*, (Oct 5, 2012).
138. K. Koshibu *et al.*, Protein phosphatase 1 regulates the histone code for long-term memory. *J Neurosci* **29**, 13079 (Oct 14, 2009).
139. S. E. File, Factors controlling measures of anxiety and responses to novelty in the mouse. *Behav Brain Res* **125**, 151 (Nov 1, 2001).
140. A. O. Mechan, A. Wyss, H. Rieger, M. H. Mohajeri, A comparison of learning and memory characteristics of young and middle-aged wild-type mice in the IntelliCage. *J Neurosci Methods* **180**, 43 (May 30, 2009).
141. L. H. Epstein, S. J. Salvy, K. A. Carr, K. K. Dearing, W. K. Bickel, Food reinforcement, delay discounting and obesity. *Physiol Behav* **100**, 438 (Jul 14, 2010).
142. V. J. Denoble, H. Begleiter, Impairment of acquisition of a DRL schedule following prolonged ethanol consumption. *Pharmacology, biochemistry, and behavior* **10**, 393 (Mar, 1979).
143. L. Schwabe, H. Schachinger, E. R. de Kloet, M. S. Oitzl, Corticosteroids operate as a switch between memory systems. *J Cogn Neurosci* **22**, 1362 (Jul, 2010).
144. S. Berger *et al.*, Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity. *Proc Natl Acad Sci U S A* **103**, 195 (Jan 3, 2006).
145. J. W. Dalley, B. J. Everitt, T. W. Robbins, Impulsivity, compulsivity, and top-down cognitive control. *Neuron* **69**, 680 (Feb 24, 2011).
146. M. Munier *et al.*, Regulation of mineralocorticoid receptor expression during neuronal differentiation of murine embryonic stem cells. *Endocrinology* **151**, 2244 (May, 2010).
147. J. Graff, B. T. Wolde Michael, D. Berchtold, G. Dewarrat, I. M. Mansuy, Dynamic histone marks in the hippocampus and cortex facilitate memory consolidation. *Nat Commun* **3**, 991 (Aug 7, 2012).
148. A. C. Hansson, K. Fuxe, Biphasic autoregulation of mineralocorticoid receptor mRNA in the medial septal nucleus by aldosterone. *Neuroendocrinology* **75**, 358 (Jun, 2002).
149. L. Daxinger, E. Whitelaw, Understanding transgenerational epigenetic inheritance via the gametes in mammals. *Nat Rev Genet* **13**, 153 (Mar, 2012).
150. C. J. Rice, C. A. Sandman, M. R. Lenjavi, T. Z. Baram, A novel mouse model for acute and long-lasting consequences of early life stress. *Endocrinology* **149**, 4892 (Oct, 2008).

151. C. Graybeal *et al.*, Paradoxical reversal learning enhancement by stress or prefrontal cortical damage: rescue with BDNF. *Nat Neurosci* **14**, 1507 (Dec, 2011).
152. T. B. Franklin, N. Linder, H. Russig, B. Thony, I. M. Mansuy, Influence of early stress on social abilities and serotonergic functions across generations in mice. *PLoS One* **6**, e21842 (2011).
153. I. C. Weiss, T. B. Franklin, S. Vizi, I. M. Mansuy, Inheritable effect of unpredictable maternal separation on behavioral responses in mice. *Front Behav Neurosci* **5**, 3 (2011).
154. N. Tsankova, W. Renthal, A. Kumar, E. J. Nestler, Epigenetic regulation in psychiatric disorders. *Nat Rev Neurosci* **8**, 355 (May, 2007).
155. R. Oliva, J. Castillo, Proteomics and the genetics of sperm chromatin condensation. *Asian journal of andrology* **13**, 24 (Jan, 2011).
156. U. Brykczynska *et al.*, Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol* **17**, 679 (Jun, 2010).
157. M. D. Klok *et al.*, Decreased expression of mineralocorticoid receptor mRNA and its splice variants in postmortem brain regions of patients with major depressive disorder. *J Psychiatr Res* **45**, 871 (Jul, 2011).
158. J. F. Lopez, D. T. Chalmers, K. Y. Little, S. J. Watson, A.E. Bennett Research Award. Regulation of serotonin1A, glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. *Biol Psychiatry* **43**, 547 (Apr 15, 1998).
159. A. Meyer-Lindenberg, H. Tost, Neural mechanisms of social risk for psychiatric disorders. *Nat Neurosci* **15**, 663 (May, 2012).
160. C. Heim, D. J. Newport, T. Mletzko, A. H. Miller, C. B. Nemeroff, The link between childhood trauma and depression: insights from HPA axis studies in humans. *Psychoneuroendocrinology* **33**, 693 (Jul, 2008).
161. A. Bifulco *et al.*, Childhood adversity, parental vulnerability and disorder: examining inter-generational transmission of risk. *Journal of child psychology and psychiatry, and allied disciplines* **43**, 1075 (Nov, 2002).
162. S. K. Sterba, M. J. Prinstein, M. J. Cox, Trajectories of internalizing problems across childhood: heterogeneity, external validity, and gender differences. *Development and psychopathology* **19**, 345 (Spring, 2007).
163. D. M. Dietz *et al.*, Paternal transmission of stress-induced pathologies. *Biological psychiatry* **70**, 408 (Sep 1, 2011).
164. A. B. Rodgers, C. P. Morgan, S. L. Bronson, S. Revello, T. L. Bale, Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**, 9003 (May 22, 2013).
165. T. B. Franklin, B. J. Saab, I. M. Mansuy, Neural mechanisms of stress resilience and vulnerability. *Neuron* **75**, 747 (Sep 6, 2012).
166. T. Y. Pang, A. J. Hannan, Enhancement of cognitive function in models of brain disease through environmental enrichment and physical activity. *Neuropharmacology* **64**, 515 (Jan, 2013).
167. J. Bohacek, K. Gapp, B. J. Saab, I. M. Mansuy, Transgenerational epigenetic effects on brain functions. *Biological Psychiatry* **73**, 313 (Feb 15, 2013).
168. M. Cui *et al.*, Enriched environment experience overcomes the memory deficits and depressive-like behavior induced by early life stress. *Neurosci Lett* **404**, 208 (Aug 14, 2006).
169. S. H. Richter, B. Zeuch, M. A. Riva, P. Gass, B. Vollmayr, Environmental enrichment ameliorates depressive-like symptoms in young rats bred for learned helplessness. *Behav Brain Res* **252**, 287 (Sep 1, 2013).
170. J. Bohacek, K. Gapp, B. J. Saab, I. M. Mansuy, Transgenerational Epigenetic Effects on Brain Functions. *Biol Psychiatry*, (Oct 9, 2012).
171. J. Simpson, D. Bree, J. P. Kelly, Effect of early life housing manipulation on baseline and drug-induced behavioural responses on neurochemistry in the male rat. *Progress in neuro-psychopharmacology & biological psychiatry* **37**, 252 (Jun 1, 2012).
172. M. E. Page, M. J. Detke, A. Dalvi, L. G. Kirby, I. Lucki, Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. *Psychopharmacology (Berl)* **147**, 162 (Nov, 1999).

173. L. MacGillivray, K. B. Reynolds, P. I. Rosebush, M. F. Mazurek, The comparative effects of environmental enrichment with exercise and serotonin transporter blockade on serotonergic neurons in the dorsal raphe nucleus. *Synapse* **66**, 465 (May, 2012).
174. Y. Chandramohan, S. K. Droste, J. S. Arthur, J. M. Reul, The forced swimming-induced behavioural immobility response involves histone H3 phospho-acetylation and c-Fos induction in dentate gyrus granule neurons via activation of the N-methyl-D-aspartate/extracellular signal-regulated kinase/mitogen- and stress-activated kinase signalling pathway. *Eur J Neurosci* **27**, 2701 (May, 2008).
175. T. Chen, Mechanistic and functional links between histone methylation and DNA methylation. *Prog Mol Biol Transl Sci* **101**, 335 (2011).
176. S. Rasmussen *et al.*, Environmental enrichment selectively increases 5-HT1A receptor mRNA expression and binding in the rat hippocampus. *Brain research. Molecular brain research* **53**, 285 (Jan, 1998).
177. I. Branchi *et al.*, Antidepressant treatment outcome depends on the quality of the living environment: a pre-clinical investigation in mice. *PLoS one* **8**, e62226 (2013).
178. A. P. West, Neurobehavioral studies of forced swimming: the role of learning and memory in the forced swim test. *Progress in neuro-psychopharmacology & biological psychiatry* **14**, 863 (1990).
179. R. D. Porsolt, M. Le Pichon, M. Jalfre, Depression: a new animal model sensitive to antidepressant treatments. *Nature* **266**, 730 (Apr 21, 1977).
180. J. M. Spivey *et al.*, Adolescent female rats are more resistant than males to the effects of early stress on prefrontal cortex and impulsive behavior. *Dev Psychobiol* **51**, 277 (Apr, 2009).
181. P. M. Wall, C. Messier, Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. *Neuroscience and biobehavioral reviews* **25**, 275 (May, 2001).
182. S. Baldini *et al.*, Enriched early life experiences reduce adult anxiety-like behavior in rats: a role for insulin-like growth factor 1. *J Neurosci* **33**, 11715 (Jul 10, 2013).
183. N. Benaroya-Milshtein *et al.*, Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity. *The European journal of neuroscience* **20**, 1341 (Sep, 2004).
184. P. Chapillon, C. Manneche, C. Belzung, J. Caston, Rearing environmental enrichment in two inbred strains of mice: 1. Effects on emotional reactivity. *Behavior genetics* **29**, 41 (Jan, 1999).
185. J. L. Workman, L. K. Fonken, J. Gusfa, K. M. Kassouf, R. J. Nelson, Post-weaning environmental enrichment alters affective responses and interacts with behavioral testing to alter nNOS immunoreactivity. *Pharmacology, biochemistry, and behavior* **100**, 25 (Nov, 2011).
186. J. E. Friske, S. C. Gammie, Environmental enrichment alters plus maze, but not maternal defense performance in mice. *Physiology & behavior* **85**, 187 (Jun 2, 2005).
187. C. Guerrero-Bosagna, M. K. Skinner, Environmentally induced epigenetic transgenerational inheritance of phenotype and disease. *Mol Cell Endocrinol* **354**, 3 (May 6, 2012).
188. ESF, ESF-EMRC Position on the Proposal for a directive on the protection of animals used for scientific purposes. (2009).
189. J. P. Curley, S. Davidson, P. Bateson, F. A. Champagne, Social enrichment during postnatal development induces transgenerational effects on emotional and reproductive behavior in mice. *Front Behav Neurosci* **3**, 25 (2009).
190. R. Mashhoodh, B. Franks, J. P. Curley, F. A. Champagne, Paternal social enrichment effects on maternal behavior and offspring growth. *Proc Natl Acad Sci U S A* **109 Suppl 2**, 17232 (Oct 16, 2012).
191. T. Fullston *et al.*, Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *The FASEB Journal*, (July 11, 2013, 2013).
192. M. V. Schmidt, X. D. Wang, O. C. Meijer, Early life stress paradigms in rodents: potential animal models of depression? *Psychopharmacology* **214**, 131 (Mar, 2011).
193. M. R. M. Richards, *The Integration of a Child into a social World*. (Cambridge University Press, New York, 1974).

194. S. R. Kaler, B. J. Freeman, Analysis of environmental deprivation: cognitive and social development in Romanian orphans. *Journal of child psychology and psychiatry, and allied disciplines* **35**, 769 (May, 1994).
195. L. McGoron *et al.*, Recovering from early deprivation: attachment mediates effects of caregiving on psychopathology. *Journal of the American Academy of Child and Adolescent Psychiatry* **51**, 683 (Jul, 2012).
196. C. Beckett *et al.*, Do the effects of early severe deprivation on cognition persist into early adolescence? Findings from the English and Romanian adoptees study. *Child development* **77**, 696 (May-Jun, 2006).
197. C. A. Nelson, 3rd *et al.*, Cognitive recovery in socially deprived young children: the Bucharest Early Intervention Project. *Science* **318**, 1937 (Dec 21, 2007).
198. M. Reuter *et al.*, The role of cortisol suppression on craving for and satisfaction from nicotine in high and low impulsive subjects. *Hum Psychopharmacol* **17**, 213 (Jul, 2002).
199. T. A. Manolio *et al.*, Finding the missing heritability of complex diseases. *Nature* **461**, 747 (Oct 8, 2009).
200. I. A. Qureshi, M. F. Mehler, Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease. *Nat Rev Neurosci* **13**, 528 (Aug, 2012).
201. M. Abe, N. M. Bonini, MicroRNAs and neurodegeneration: role and impact. *Trends in cell biology* **23**, 30 (Jan, 2013).
202. V. Rottiers, A. M. Naar, MicroRNAs in metabolism and metabolic disorders. *Nature reviews. Molecular cell biology* **13**, 239 (Apr, 2012).
203. N. O. Burton, K. B. Burkhardt, S. Kennedy, Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* **108**, 19683 (Dec 6, 2011).
204. S. G. Gu *et al.*, Amplification of siRNA in *Caenorhabditis elegans* generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. *Nat Genet* **44**, 157 (Feb, 2012).
205. W. M. Liu *et al.*, Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc Natl Acad Sci U S A* **109**, 490 (Jan 10, 2012).
206. M. Kawano, H. Kawaji, V. Grandjean, J. Kiani, M. Rassoulzadegan, Novel small noncoding RNAs in mouse spermatozoa, zygotes and early embryos. *PloS one* **7**, e44542 (2012).
207. S. A. Krawetz *et al.*, A survey of small RNAs in human sperm. *Hum Reprod* **26**, 3401 (Dec, 2011).
208. G. D. Johnson *et al.*, Cleavage of rRNA ensures translational cessation in sperm at fertilization. *Mol Hum Reprod* **17**, 721 (Dec, 2011).
209. F. J. Pena *et al.*, Mitochondria in mammalian sperm physiology and pathology: a review. *Reproduction in domestic animals = Zuchthygiene* **44**, 345 (Apr, 2009).
210. S. Pellow, P. Chopin, S. E. File, M. Briley, Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* **14**, 149 (Aug, 1985).
211. J. Crawley, F. K. Goodwin, Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacology, biochemistry, and behavior* **13**, 167 (Aug, 1980).
212. G. J. Boersma, S. R. Salton, P. M. Spritzer, C. T. Steele, D. L. Carbone, Models and mechanisms of metabolic regulation: genes, stress, and the HPA and HPG axes. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* **44**, 598 (Jul, 2012).
213. M. Maragakis *et al.*, DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association. *Nucleic Acids Res* **39**, W145 (Jul, 2011).
214. B. S. McEwen, Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. *Annals of the New York Academy of Sciences* **1204 Suppl**, E38 (Sep, 2010).
215. A. El Ouamari *et al.*, miR-375 targets 3'-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic beta-cells. *Diabetes* **57**, 2708 (Oct, 2008).
216. M. N. Poy *et al.*, A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432**, 226 (Nov 11, 2004).

217. K. Abdelmohsen *et al.*, miR-375 inhibits differentiation of neurites by lowering HuD levels. *Molecular and cellular biology* **30**, 4197 (Sep, 2010).
218. T. D. Gould *et al.*, Generation and behavioral characterization of beta-catenin forebrain-specific conditional knock-out mice. *Behav Brain Res* **189**, 117 (May 16, 2008).
219. Y. C. Chen *et al.*, The effect of citalopram on chronic stress-induced depressive-like behavior in rats through GSK3beta/beta-catenin activation in the medial prefrontal cortex. *Brain research bulletin* **88**, 338 (Jul 1, 2012).
220. S. Haramati *et al.*, MicroRNA as repressors of stress-induced anxiety: the case of amygdalar miR-34. *J Neurosci* **31**, 14191 (Oct 5, 2011).
221. A. Meerson *et al.*, Changes in brain MicroRNAs contribute to cholinergic stress reactions. *Journal of molecular neuroscience : MN* **40**, 47 (Jan, 2010).
222. A. Rinaldi *et al.*, Stress induces region specific alterations in microRNAs expression in mice. *Behav Brain Res* **208**, 265 (Mar 17, 2010).
223. N. Zhang *et al.*, MicroRNA 375 mediates the signaling pathway of corticotropin-releasing factor (CRF) regulating pro-opiomelanocortin (POMC) expression by targeting mitogen-activated protein kinase 8. *The Journal of biological chemistry* **288**, 10361 (Apr 12, 2013).
224. K. A. Maguschak, K. J. Ressler, A role for WNT/beta-catenin signaling in the neural mechanisms of behavior. *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* **7**, 763 (Dec, 2012).
225. M. Maragkakis *et al.*, Accurate microRNA target prediction correlates with protein repression levels. *BMC bioinformatics* **10**, 295 (2009).
226. B. C. Hogan, F. and Lacy, L., *Manipulating the Mouse Embryo - A Laboratory manual, Second Edition*. (Cold Spring Harbor Laboratory, NY, 1994).
227. S. Sai Lakshmi, S. Agrawal, piRNABank: a web resource on classified and clustered Piwi-interacting RNAs. *Nucleic Acids Res* **36**, D173 (Jan, 2008).
228. H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754 (Jul 15, 2009).
229. A. Kozomara, S. Griffiths-Jones, miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res* **39**, D152 (Jan, 2011).
230. S. Anders, W. Huber, Differential expression analysis for sequence count data. *Genome biology* **11**, R106 (2010).
231. D. Betel, R. Sheridan, D. S. Marks, C. Sander, Computational analysis of mouse piRNA sequence and biogenesis. *PLoS computational biology* **3**, e222 (Nov, 2007).
232. B. Langmead, C. Trapnell, M. Pop, S. L. Salzberg, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology* **10**, R25 (2009).
233. E. Dias-Ferreira *et al.*, Chronic stress causes frontostriatal reorganization and affects decision-making. *Science* **325**, 621 (Jul 31, 2009).
234. T. J. Shors, Stressful experience and learning across the lifespan. *Annual review of psychology* **57**, 55 (2006).
235. B. S. McEwen, Stress, adaptation, and disease. Allostasis and allostatic load. *Annals of the New York Academy of Sciences* **840**, 33 (May 1, 1998).
236. M. Manikkam, R. Tracey, C. Guerrero-Bosagna, M. K. Skinner, Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. *PloS one* **7**, e46249 (2012).
237. M. Manikkam, R. Tracey, C. Guerrero-Bosagna, M. K. Skinner, Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PloS one* **8**, e55387 (2013).
238. J. D. Hollister, B. S. Gaut, Epigenetic silencing of transposable elements: a trade-off between reduced transposition and deleterious effects on neighboring gene expression. *Genome research* **19**, 1419 (Aug, 2009).
239. J. Li *et al.*, Genomic hypomethylation in the human germline associates with selective structural mutability in the human genome. *PLoS Genet* **8**, e1002692 (2012).
240. E. L. Greer *et al.*, Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*. *Nature* **479**, 365 (Nov 17, 2011).

241. D. H. Kim, P. Saetrom, O. Snove, Jr., J. J. Rossi, MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A* **105**, 16230 (Oct 21, 2008).
242. G. D. Johnson *et al.*, The sperm nucleus: chromatin, RNA, and the nuclear matrix. *Reproduction* **141**, 21 (Jan, 2011).
243. C. Lalancette, D. Miller, Y. Li, S. A. Krawetz, Paternal contributions: new functional insights for spermatozoal RNA. *J Cell Biochem* **104**, 1570 (Aug 1, 2008).
244. D. M. Dietz *et al.*, Paternal transmission of stress-induced pathologies. *Biological psychiatry* **70**, 408 (Sep 1, 2011).
245. D. Liu *et al.*, Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* **277**, 1659 (Sep 12, 1997).
246. P. M. Plotsky, M. J. Meaney, Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Brain research. Molecular brain research* **18**, 195 (May, 1993).
247. C. R. Pryce *et al.*, Long-term effects of early-life environmental manipulations in rodents and primates: Potential animal models in depression research. *Neuroscience and biobehavioral reviews* **29**, 649 (2005).
248. M. M. Sanchez, C. O. Ladd, P. M. Plotsky, Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. *Development and psychopathology* **13**, 419 (Summer, 2001).
249. E. R. de Kloet, R. M. Sibug, F. M. Helmerhorst, M. V. Schmidt, Stress, genes and the mechanism of programming the brain for later life. *Neuroscience and biobehavioral reviews* **29**, 271 (Apr, 2005).
250. S. M. Southwick, D. S. Charney, The science of resilience: implications for the prevention and treatment of depression. *Science* **338**, 79 (Oct 5, 2012).
251. T. Liu *et al.*, Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis* **28**, 488 (Feb, 2007).
252. H. R. Kim, K. A. Hwang, K. C. Kim, I. Kang, Down-regulation of IL-7Ralpha expression in human T cells via DNA methylation. *J Immunol* **178**, 5473 (May 1, 2007).
253. B. R. Mueller, T. L. Bale, Sex-specific programming of offspring emotionality after stress early in pregnancy. *J Neurosci* **28**, 9055 (Sep 3, 2008).
254. A. A. Zozulya, M. V. Gabaeva, O. Y. Sokolov, I. D. Surkina, N. V. Kost, Personality, coping style, and constitutional neuroimmunology. *Journal of immunotoxicology* **5**, 221 (Apr, 2008).
255. R. Jankord, J. P. Herman, Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. *Annals of the New York Academy of Sciences* **1148**, 64 (Dec, 2008).
256. E. Roman, L. Gustafsson, M. Berg, I. Nylander, Behavioral profiles and stress-induced corticosteroid secretion in male Wistar rats subjected to short and prolonged periods of maternal separation. *Hormones and behavior* **50**, 736 (Dec, 2006).
257. K. M. Hutchinson *et al.*, Environmental enrichment protects against the effects of chronic stress on cognitive and morphological measures of hippocampal integrity. *Neurobiology of learning and memory* **97**, 250 (Feb, 2012).
258. S. M. Korte, Corticosteroids in relation to fear, anxiety and psychopathology. *Neuroscience and biobehavioral reviews* **25**, 117 (Mar, 2001).
259. L. M. Villeneuve *et al.*, Enhanced levels of microRNA-125b in vascular smooth muscle cells of diabetic db/db mice lead to increased inflammatory gene expression by targeting the histone methyltransferase Suv39h1. *Diabetes* **59**, 2904 (Nov, 2010).
260. L. M. Villeneuve, M. A. Reddy, R. Natarajan, Epigenetics: deciphering its role in diabetes and its chronic complications. *Clin Exp Pharmacol Physiol* **38**, 401 (Jul, 2011).
261. A. P. Wolffe, M. A. Matzke, Epigenetics: Regulation Through Repression. *Science* **286**, 481 (October 15, 1999, 1999).
262. S. R. Kinney, S. Pradhan, Regulation of expression and activity of DNA (cytosine-5) methyltransferases in mammalian cells. *Prog Mol Biol Transl Sci* **101**, 311 (2011).
263. M. N. Davies *et al.*, Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome biology* **13**, R43 (2012).

264. A. Bird, DNA methylation patterns and epigenetic memory. *Genes & development* **16**, 6 (Jan 1, 2002).
265. M. Esteller, Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* **8**, 286 (2007).
266. R. J. Klose, A. P. Bird, Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* **31**, 89 (Feb, 2006).
267. D. H. Yasui *et al.*, Integrated epigenomic analyses of neuronal MeCP2 reveal a role for long-range interaction with active genes. *Proc Natl Acad Sci U S A* **104**, 19416 (Dec 4, 2007).
268. M. Chahrour *et al.*, MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* **320**, 1224 (May 30, 2008).
269. P. B. Talbert, S. Henikoff, Histone variants--ancient wrap artists of the epigenome. *Nature reviews. Molecular cell biology* **11**, 264 (Apr, 2010).
270. R. Y. Tweedie-Cullen *et al.*, Identification of combinatorial patterns of post-translational modifications on individual histones in the mouse brain. *PloS one* **7**, e36980 (2012).
271. V. W. Zhou, A. Goren, B. E. Bernstein, Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet* **12**, 7 (Jan, 2011).
272. M. R. Branco, G. Ficz, W. Reik, Uncovering the role of 5-hydroxymethylcytosine in the epigenome. *Nat Rev Genet* **15**, (Nov 15, 2011).
273. M. U. Kaikkonen, M. T. Lam, C. K. Glass, Non-coding RNAs as regulators of gene expression and epigenetics. *Cardiovasc Res* **90**, 430 (Jun 1, 2011).
274. J. Gräff, I. M. Mansuy, Epigenetic codes in cognition and behaviour. *Behav Brain Res* **192**, 70 (Sep 1, 2008).
275. J. Feng, S. Fouse, G. Fan, Epigenetic regulation of neural gene expression and neuronal function. *Pediatric research* **61**, 58R (May, 2007).
276. Y. Hirabayashi, Y. Gotoh, Epigenetic control of neural precursor cell fate during development. *Nat Rev Neurosci* **11**, 377 (Jun, 2010).
277. C. Y. Brazel, M. J. Romanko, R. P. Rothstein, S. W. Levison, Roles of the mammalian subventricular zone in brain development. *Prog Neurobiol* **69**, 49 (Jan, 2003).
278. A. P. van Montfoort *et al.*, Assisted reproduction treatment and epigenetic inheritance. *Hum Reprod Update* **18**, 171 (Mar-Apr, 2012).
279. N. Ballas, C. Grunseich, D. D. Lu, J. C. Speh, G. Mandel, REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell* **121**, 645 (May 20, 2005).
280. V. V. Lunyak *et al.*, Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* **298**, 1747 (Nov 29, 2002).
281. M. Sikorska *et al.*, Epigenetic modifications of SOX2 enhancers, SRR1 and SRR2, correlate with in vitro neural differentiation. *J Neurosci Res* **86**, 1680 (Jun, 2008).
282. Y. Sun *et al.*, Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* **104**, 365 (Feb 9, 2001).
283. T. Takizawa *et al.*, DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Dev Cell* **1**, 749 (Dec, 2001).
284. M. Namihira, K. Nakashima, T. Taga, Developmental stage dependent regulation of DNA methylation and chromatin modification in a immature astrocyte specific gene promoter. *FEBS Lett* **572**, 184 (Aug 13, 2004).
285. G. Fan *et al.*, DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. *Development* **132**, 3345 (Aug, 2005).
286. K. Goto *et al.*, Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* **56**, 39 (Apr, 1994).
287. J. Feng *et al.*, Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nature neuroscience* **13**, 423 (April, 2010).
288. P. J. Brooks, C. Marietta, D. Goldman, DNA mismatch repair and DNA methylation in adult brain neurons. *J Neurosci* **16**, 939 (Feb 1, 1996).
289. L. K. Hutnick *et al.*, DNA hypomethylation restricted to the murine forebrain induces cortical degeneration and impairs postnatal neuronal maturation. *Human molecular genetics* **18**, 2875 (Aug 1, 2009).
290. H. Wu *et al.*, Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. *Science* **329**, 444 (Jul 23, 2010).
291. Z. Wu *et al.*, Dnmt3a regulates both proliferation and differentiation of mouse neural stem cells. *J Neurosci Res* **90**, 1883 (Oct, 2012).

292. J. Feng, H. Chang, E. Li, G. Fan, Dynamic expression of de novo DNA methyltransferases Dnmt3a and Dnmt3b in the central nervous system. *J Neurosci Res* **79**, 734 (Mar 15, 2005).
293. D. Watanabe, K. Uchiyama, K. Hanaoka, Transition of mouse de novo methyltransferases expression from Dnmt3b to Dnmt3a during neural progenitor cell development. *Neuroscience* **142**, 727 (Oct 27, 2006).
294. S. Bai *et al.*, DNA methyltransferase 3b regulates nerve growth factor-induced differentiation of PC12 cells by recruiting histone deacetylase 2. *Molecular and cellular biology* **25**, 751 (Jan, 2005).
295. E. R. Lee, F. E. Murdoch, M. K. Fritsch, High histone acetylation and decreased polycomb repressive complex 2 member levels regulate gene specific transcriptional changes during early embryonic stem cell differentiation induced by retinoic acid. *Stem Cells* **25**, 2191 (Sep, 2007).
296. T. S. Mikkelsen *et al.*, Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* **448**, 553 (Aug 2, 2007).
297. T. Burgold *et al.*, The histone H3 lysine 27-specific demethylase Jmjd3 is required for neural commitment. *PLoS one* **3**, e3034 (2008).
298. M. Attia, C. Rachez, A. De Pauw, P. Avner, U. C. Rogner, Nap1l2 promotes histone acetylation activity during neuronal differentiation. *Molecular and cellular biology* **27**, 6093 (Sep, 2007).
299. M. Marin-Husstege, M. Muggironi, A. Liu, P. Casaccia-Bonelli, Histone deacetylase activity is necessary for oligodendrocyte lineage progression. *J Neurosci* **22**, 10333 (Dec 1, 2002).
300. J. Hsieh, K. Nakashima, T. Kuwabara, E. Mejia, F. H. Gage, Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci U S A* **101**, 16659 (Nov 23, 2004).
301. C. A. Lyssiotis *et al.*, Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. *Proc Natl Acad Sci U S A* **104**, 14982 (Sep 18, 2007).
302. Y. He, J. Sandoval, P. Casaccia-Bonelli, Events at the transition between cell cycle exit and oligodendrocyte progenitor differentiation: the role of HDAC and YY1. *Neuron Glia Biol* **3**, 221 (Aug, 2007).
303. S. Shen, J. Li, P. Casaccia-Bonelli, Histone modifications affect timing of oligodendrocyte progenitor differentiation in the developing rat brain. *J Cell Biol* **169**, 577 (May 23, 2005).
304. J. Morrens, W. Van Den Broeck, G. Kempermann, Glial cells in adult neurogenesis. *Glia* **60**, 159 (Feb, 2012).
305. G. L. Ming, H. Song, Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* **70**, 687 (May 26, 2011).
306. M. S. Zainuddin, S. Thuret, Nutrition, adult hippocampal neurogenesis and mental health. *Br Med Bull* **103**, 89 (2012).
307. K. Fabel *et al.*, Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis in mice. *Front Neurosci* **3**, 50 (2009).
308. S. A. Wolf, A. Melnik, G. Kempermann, Physical exercise increases adult neurogenesis and telomerase activity, and improves behavioral deficits in a mouse model of schizophrenia. *Brain, behavior, and immunity* **25**, 971 (Jul, 2011).
309. H. van Praag, T. Shubert, C. Zhao, F. H. Gage, Exercise enhances learning and hippocampal neurogenesis in aged mice. *J Neurosci* **25**, 8680 (Sep 21, 2005).
310. D. K. Ma *et al.*, Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. *Science* **323**, 1074 (Feb 20, 2009).
311. Z. Gao *et al.*, The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells. *J Neurosci* **31**, 9772 (Jun 29, 2011).
312. D. A. Lim *et al.*, Chromatin remodelling factor Mll1 is essential for neurogenesis from postnatal neural stem cells. *Nature* **458**, 529 (Mar 26, 2009).
313. C. A. Fasano *et al.*, Bmi-1 cooperates with Foxg1 to maintain neural stem cell self-renewal in the forebrain. *Genes & development* **23**, 561 (Mar 1, 2009).
314. K. E. Szulwach *et al.*, Cross talk between microRNA and epigenetic regulation in adult neurogenesis. *J Cell Biol* **189**, 127 (Apr 5, 2010).

315. C. Liu *et al.*, Epigenetic regulation of miR-184 by MBD1 governs neural stem cell proliferation and differentiation. *Cell Stem Cell* **6**, 433 (May 7, 2010).
316. e. a. Lu Tao, Gene regulation and DNA damage in the ageing human brain. *Nature* **429**, 883 (24 June 2004, 2004).
317. B. A. Yankner, T. Lu, P. Loerch, The aging brain. *Annu Rev Pathol* **3**, 41 (2008).
318. H. J. Kang *et al.*, Spatio-temporal transcriptome of the human brain. *Nature* **478**, 483 (Oct 27, 2011).
319. S. H. Wood, T. Craig, Y. Li, B. Merry, J. P. de Magalhaes, Whole transcriptome sequencing of the aging rat brain reveals dynamic RNA changes in the dark matter of the genome. *Age (Dordr)* **4**, 4 (May 4, 2012).
320. C. K.-C. Blalock Eric M, Sharroo Keith, Herman James P., Porter Nada, Foster Thomas, Landfield Philip, Gene Microarrays in Hippocampal Aging: Statistical Profiling Identifies Novel Processes Correlated with Cognitive Impairment. *The Journal of Neuroscience* **23**, 3807 (2003).
321. e. a. Rowe Wayne B., Hippocampal expression analyses reveal selective association of immediate-early, neuroenergetic, and myelinogenic pathways with cognitive impairment in aged rats. *Journal of Neuroscience* **27**, 3098 (2007).
322. K. D. Siegmund *et al.*, DNA methylation in the human cerebral cortex is dynamically regulated throughout the life span and involves differentiated neurons. *PLoS one* **2**, e895 (2007).
323. D. G. Hernandez *et al.*, Distinct DNA methylation changes highly correlated with chronological age in the human brain. *Human molecular genetics* **20**, 1164 (Mar 15, 2011).
324. C. A. Miller *et al.*, Cortical DNA methylation maintains remote memory. *Nat Neurosci* **13**, 664 (Jun, 2010).
325. J. Gräff, B. Woldemichael, D. Berchtold, D. G. M. I, Dynamic histone marks in the hippocampus and cortex facilitate memory consolidation. *Nature Communications* **in press**, (2012).
326. M. R. Penner *et al.*, Age-related changes in Arc transcription and DNA methylation within the hippocampus. *Neurobiology of aging* **32**, 2198 (Dec, 2011).
327. W. B. Rowe *et al.*, Hippocampal expression analyses reveal selective association of immediate-early, neuroenergetic, and myelinogenic pathways with cognitive impairment in aged rats. *J Neurosci* **27**, 3098 (Mar 21, 2007).
328. S. Peleg *et al.*, Altered histone acetylation is associated with age-dependent memory impairment in mice. *Science* **328**, 753 (May 7, 2010).
329. A. Fischer, F. Sananbenesi, X. Wang, M. Dobbin, L. H. Tsai, Recovery of learning and memory is associated with chromatin remodelling. *Nature* **447**, 178 (May 10, 2007).
330. J. F. Castellano *et al.*, Age-related memory impairment is associated with disrupted multivariate epigenetic coordination in the hippocampus. *PLoS one* **7**, e33249 (2012).
331. K. Sha, A mechanistic view of genomic imprinting. *Annu Rev Genomics Hum Genet* **9**, 197 (2008).
332. A. Paoloni-Giacobino, J. Chaillet, The role of DMDs in the maintenance of epigenetic states. *Cytogenet Genome Res* **113**, 116 (2006).
333. S. J. Chamberlain, M. Lalande, Neurodevelopmental disorders involving genomic imprinting at human chromosome 15q11-q13. *Neurobiology of Disease* **39**, 13.
334. S. J. Chamberlain, M. Lalande, Neurodevelopmental disorders involving genomic imprinting at human chromosome 15q11-q13. *Neurobiol Dis* **39**, 13 (Jul, 2010).
335. R. Weksberg, C. Shuman, J. B. Beckwith, Beckwith-Wiedemann syndrome. *European Journal of Human Genetics* **18**, 8 (2010).
336. S. J. Chamberlain, M. Lalande, Angelman Syndrome, a Genomic Imprinting Disorder of the Brain. *J. Neurosci.* **30**, 9958 (July 28, 2010).
337. S. B. Cassidy, E. Dykens, C. A. Williams, Prader-Willi and Angelman syndromes: Sister imprinted disorders. *American Journal of Medical Genetics* **97**, 136 (2000).
338. K. N. Leung, S. J. Chamberlain, M. Lalande, J. M. LaSalle, Neuronal chromatin dynamics of imprinting in development and disease. *J Cell Biochem* **112**, 365 (Feb, 2011).
339. R. E. Amir *et al.*, Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet* **23**, 185 (Oct, 1999).
340. M. Chahrour, H. Y. Zoghbi, The story of Rett syndrome: from clinic to neurobiology. *Neuron* **56**, 422 (Nov 8, 2007).

341. S. Ben-Shachar, M. Chahrour, C. Thaller, C. A. Shaw, H. Y. Zoghbi, Mouse models of MeCP2 disorders share gene expression changes in the cerebellum and hypothalamus. *Human molecular genetics* **18**, 2431 (Jul 1, 2009).
342. A. L. Collins *et al.*, Mild overexpression of MeCP2 causes a progressive neurological disorder in mice. *Human molecular genetics* **13**, 2679 (Nov 1, 2004).
343. R. Z. Chen, S. Akbarian, M. Tudor, R. Jaenisch, Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet* **27**, 327 (Mar, 2001).
344. N. Ballas, D. T. Lioy, C. Grunseich, G. Mandel, Non-cell autonomous influence of MeCP2-deficient glia on neuronal dendritic morphology. *Nat Neurosci* **12**, 311 (Mar, 2009).
345. I. Maezawa, S. Swanberg, D. Harvey, J. M. LaSalle, L. W. Jin, Rett syndrome astrocytes are abnormal and spread MeCP2 deficiency through gap junctions. *J Neurosci* **29**, 5051 (Apr 22, 2009).
346. D. T. Lioy *et al.*, A role for glia in the progression of Rett's syndrome. *Nature* **475**, 497 (Jul 28, 2011).
347. J. Guy, B. Hendrich, M. Holmes, J. E. Martin, A. Bird, A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet* **27**, 322 (Mar, 2001).
348. R. C. Samaco *et al.*, Female Mecp2 $^{+/-}$  mice display robust behavioral deficits on two different genetic backgrounds providing a framework for pre-clinical studies. *Human molecular genetics*, (Oct 1, 2012).
349. W. Li, G. Calfa, J. Larimore, L. Pozzo-Miller, Activity-dependent BDNF release and TRPC signaling is impaired in hippocampal neurons of Mecp2 mutant mice. *Proc Natl Acad Sci U S A*, (Oct 1, 2012).
350. M. P. Blackman, B. Djukic, S. B. Nelson, G. G. Turrigiano, A Critical and Cell-Autonomous Role for MeCP2 in Synaptic Scaling Up. *J Neurosci* **32**, 13529 (Sep 26, 2012).
351. J. Guy, J. Gan, J. Selfridge, S. Cobb, A. Bird, Reversal of neurological defects in a mouse model of Rett syndrome. *Science* **315**, 1143 (Feb 23, 2007).
352. D. Tropea *et al.*, Partial reversal of Rett Syndrome-like symptoms in MeCP2 mutant mice. *Proc Natl Acad Sci U S A* **106**, 2029 (Feb 10, 2009).
353. M. Tudor, S. Akbarian, R. Z. Chen, R. Jaenisch, Transcriptional profiling of a mouse model for Rett syndrome reveals subtle transcriptional changes in the brain. *Proc Natl Acad Sci U S A* **99**, 15536 (Nov 26, 2002).
354. R. G. Urdinguio *et al.*, Mecp2-null mice provide new neuronal targets for Rett syndrome. *PLoS one* **3**, e3669 (2008).
355. M. L. Gonzales, S. Adams, K. W. Dunaway, J. M. LaSalle, Phosphorylation of distinct sites in MeCP2 modifies cofactor associations and the dynamics of transcriptional regulation. *Molecular and cellular biology* **32**, 2894 (Jul, 2012).
356. W. G. Chen *et al.*, Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* **302**, 885 (Oct 31, 2003).
357. K. Martinowich *et al.*, DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* **302**, 890 (Oct 31, 2003).
358. R. D. Smrt *et al.*, Mecp2 deficiency leads to delayed maturation and altered gene expression in hippocampal neurons. *Neurobiol Dis* **27**, 77 (Jul, 2007).
359. Z. Zhou *et al.*, Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. *Neuron* **52**, 255 (Oct 19, 2006).
360. S. Cohen *et al.*, Genome-wide activity-dependent MeCP2 phosphorylation regulates nervous system development and function. *Neuron* **72**, 72 (Oct 6, 2011).
361. R. J. Klose, A. P. Bird, MeCP2 behaves as an elongated monomer that does not stably associate with the Sin3a chromatin remodeling complex. *The Journal of biological chemistry* **279**, 46490 (Nov 5, 2004).
362. F. Fuks *et al.*, The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *The Journal of biological chemistry* **278**, 4035 (Feb 7, 2003).
363. D. Su, Y. M. Cha, A. E. West, Mutation of MeCP2 alters transcriptional regulation of select immediate-early genes. *Epigenetics* **7**, 146 (Feb, 2012).
364. R. G. Urdinguio *et al.*, Disrupted microRNA expression caused by Mecp2 loss in a mouse model of Rett syndrome. *Epigenetics* **5**, 656 (Oct 1, 2010).

365. M. F. Fraga *et al.*, Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* **102**, 10604 (Jul 26, 2005).
366. J. M. Silverman, G. Ciresi, C. J. Smith, D. B. Marin, M. Schnaider-Beeri, Variability of familial risk of Alzheimer disease across the late life span. *Arch Gen Psychiatry* **62**, 565 (May, 2005).
367. K. L. Brickell *et al.*, Clinicopathological concordance and discordance in three monozygotic twin pairs with familial Alzheimer's disease. *J Neurol Neurosurg Psychiatry* **78**, 1050 (Oct, 2007).
368. C. Ptak, A. Petronis, Epigenetics and complex disease: from etiology to new therapeutics. *Annu Rev Pharmacol Toxicol* **48**, 257 (2008).
369. D. K. Lahiri, B. Maloney, N. H. Zawia, The LEARN model: an epigenetic explanation for idiopathic neurobiological diseases. *Mol Psychiatry* **14**, 992 (Nov, 2009).
370. B. P. Rutten, J. Mill, Epigenetic mediation of environmental influences in major psychotic disorders. *Schizophr Bull* **35**, 1045 (Nov, 2009).
371. J. Bohacek, I. M. Mansuy, Epigenetic Inheritance of Disease and Disease Risk. *Neuropsychopharmacology*, (Jul 11, 2012).
372. L. Crews, E. Masliah, Molecular mechanisms of neurodegeneration in Alzheimer's disease. *Human molecular genetics* **19**, R12 (Apr 15, 2010).
373. D. M. Niedowicz, P. T. Nelson, M. P. Murphy, Alzheimer's disease: pathological mechanisms and recent insights. *Curr Neuropharmacol* **9**, 674 (Dec, 2011).
374. N. Brouwers, K. Sleegers, C. Van Broeckhoven, Molecular genetics of Alzheimer's disease: an update. *Ann Med* **40**, 562 (2008).
375. L. Bertram, C. M. Lill, R. E. Tanzi, The genetics of Alzheimer disease: back to the future. *Neuron* **68**, 270 (Oct 21, 2010).
376. C. Cruchaga *et al.*, Rare variants in APP, PSEN1 and PSEN2 increase risk for AD in late-onset Alzheimer's disease families. *PloS one* **7**, e31039 (2012).
377. C. L. Ulrey, L. Liu, L. G. Andrews, T. O. Tollesbol, The impact of metabolism on DNA methylation. *Human molecular genetics* **14**, R139 (15 April 2005, 2005).
378. J. Lundberg *et al.*, Traumatic brain injury induces relocalization of DNA-methyltransferase 1. *Neurosci Lett* **457**, 8 (Jun 19, 2009).
379. A. S. Siersma, D. L. van den Hove, H. W. Steinbusch, J. Prickaerts, Major depression, cognitive dysfunction and Alzheimer's disease: is there a link? *Eur J Pharmacol* **626**, 72 (Jan 10, 2009).
380. D. Mastroeni *et al.*, Epigenetic changes in Alzheimer's disease: decrements in DNA methylation. *Neurobiology of aging* **31**, 2025 (Dec, 2010).
381. G. S. Philip De Jager, Matthew Eaton, Lori Chibnik, Manolis Kellis and David Bennett, in *Neurology April 25, 2012; 78(Meeting Abstracts 1): P05.070.* (2012).
382. V. Bollati *et al.*, DNA methylation in repetitive elements and Alzheimer disease. *Brain, behavior, and immunity* **25**, 1078 (Aug, 2011).
383. S. C. Wang, B. Oelze, A. Schumacher, Age-specific epigenetic drift in late-onset Alzheimer's disease. *PloS one* **3**, e2698 (2008).
384. J. Wu *et al.*, Alzheimer's disease (AD)-like pathology in aged monkeys after infantile exposure to environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD. *J Neurosci* **28**, 3 (Jan 2, 2008).
385. M. J. Gunzburg, M. A. Perugini, G. J. Howlett, Structural basis for the recognition and cross-linking of amyloid fibrils by human apolipoprotein E. *The Journal of biological chemistry* **282**, 35831 (Dec 7, 2007).
386. N. Iwata *et al.*, Identification of the major Abeta1-42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. *Nat Med* **6**, 143 (Feb, 2000).
387. K. Yasojima, H. Akiyama, E. G. McGeer, P. L. McGeer, Reduced neprilysin in high plaque areas of Alzheimer brain: a possible relationship to deficient degradation of beta-amyloid peptide. *Neurosci Lett* **297**, 97 (Jan 12, 2001).
388. X. Cao, T. C. Sudhof, A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. *Science* **293**, 115 (Jul 6, 2001).
389. P. Marambaud *et al.*, A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell* **114**, 635 (Sep 5, 2003).

390. D. Kim *et al.*, SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. *EMBO J* **26**, 3169 (Jul 11, 2007).
391. C. Rouaux *et al.*, Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration. *EMBO J* **22**, 6537 (Dec 15, 2003).
392. C. A. Saura *et al.*, Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. *Neuron* **42**, 23 (Apr 8, 2004).
393. Y. I. Francis *et al.*, Dysregulation of histone acetylation in the APP/PS1 mouse model of Alzheimer's disease. *J Alzheimers Dis* **18**, 131 (2009).
394. P. Narayan, M. Dragunow, Pharmacology of epigenetics in brain disorders. *Br J Pharmacol* **159**, 285 (Jan 1, 2010).
395. R. Berni Canani, M. Di Costanzo, L. Leone, The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. *Clin Epigenetics* **4**, 4 (2012).
396. T. Alberio, L. Lopiano, M. Fasano, Cellular models to investigate biochemical pathways in Parkinson's disease. *FEBS J* **279**, 1146 (Apr, 2012).
397. A. E. Lang, J. A. Obeso, Challenges in Parkinson's disease: restoration of the nigrostriatal dopamine system is not enough. *Lancet Neurol* **3**, 309 (May, 2004).
398. H. Braak *et al.*, Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of aging* **24**, 197 (Mar-Apr, 2003).
399. R. G. Urdinguio, J. V. Sanchez-Mut, M. Esteller, Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. *Lancet Neurol* **8**, 1056 (Nov, 2009).
400. M. J. Devine *et al.*, Pathogenic LRRK2 mutations do not alter gene expression in cell model systems or human brain tissue. *PLoS one* **6**, e22489 (2011).
401. F. Coppede, Genetics and epigenetics of Parkinson's disease. *ScientificWorldJournal* **2012**, 489830 (2012).
402. A. Elbaz, F. Moisan, [Parkinson's disease: Is there a strong environmental contribution?]. *Rev Neurol (Paris)* **166**, 757 (Oct, 2010).
403. C. M. Tanner *et al.*, Parkinson disease in twins: an etiologic study. *JAMA* **281**, 341 (Jan 27, 1999).
404. A. Jowaed, I. Schmitt, O. Kaut, U. Wullner, Methylation regulates alpha-synuclein expression and is decreased in Parkinson's disease patients' brains. *J Neurosci* **30**, 6355 (May 5, 2010).
405. G. E. Voutsinas *et al.*, Allelic imbalance of expression and epigenetic regulation within the alpha-synuclein wild-type and p.Ala53Thr alleles in Parkinson disease. *Hum Mutat* **31**, 685 (Jun, 2010).
406. E. Kontopoulos, J. D. Parvin, M. B. Feany, Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Human molecular genetics* **15**, 3012 (Oct 15, 2006).
407. T. F. Outeiro *et al.*, Sirtuin 2 inhibitors rescue alpha-synuclein-mediated toxicity in models of Parkinson's disease. *Science* **317**, 516 (Jul 27, 2007).
408. M. Mogi *et al.*, Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile parkinsonism and Parkinson's disease. *Neurosci Lett* **211**, 13 (Jun 14, 1996).
409. H. C. Pieper *et al.*, Different methylation of the TNF-alpha promoter in cortex and substantia nigra: Implications for selective neuronal vulnerability. *Neurobiol Dis* **32**, 521 (Dec, 2008).
410. A. P. Nicholas *et al.*, Striatal histone modifications in models of levodopa-induced dyskinesia. *Journal of neurochemistry* **106**, 486 (Jul, 2008).
411. G. P. Bates, History of genetic disease: the molecular genetics of Huntington disease - a history. *Nat Rev Genet* **6**, 766 (Oct, 2005).
412. F. He, P. K. Todd, Epigenetics in nucleotide repeat expansion disorders. *Semin Neurol* **31**, 470 (Nov, 2011).
413. E. C. Stack *et al.*, Modulation of nucleosome dynamics in Huntington's disease. *Human molecular genetics* **16**, 1164 (May 15, 2007).
414. J. S. Steffan *et al.*, The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc Natl Acad Sci U S A* **97**, 6763 (Jun 6, 2000).

415. K. L. Sugars, R. Brown, L. J. Cook, J. Swartz, D. C. Rubinsztein, Decreased cAMP response element-mediated transcription: an early event in exon 1 and full-length cell models of Huntington's disease that contributes to polyglutamine pathogenesis. *The Journal of biological chemistry* **279**, 4988 (Feb 6, 2004).
416. R. J. Ferrante *et al.*, Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J Neurosci* **23**, 9418 (Oct 15, 2003).
417. G. Gardian *et al.*, Neuroprotective effects of phenylbutyrate in the N171-82Q transgenic mouse model of Huntington's disease. *The Journal of biological chemistry* **280**, 556 (Jan 7, 2005).
418. E. Hockly *et al.*, Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc Natl Acad Sci U S A* **100**, 2041 (Feb 18, 2003).
419. G. Sadri-Vakili *et al.*, Histones associated with downregulated genes are hypoacetylated in Huntington's disease models. *Human molecular genetics* **16**, 1293 (Jun 1, 2007).
420. E. A. Thomas *et al.*, The HDAC inhibitor 4b ameliorates the disease phenotype and transcriptional abnormalities in Huntington's disease transgenic mice. *Proc Natl Acad Sci U S A* **105**, 15564 (Oct 7, 2008).
421. I. S. Seong *et al.*, Huntingtin facilitates polycomb repressive complex 2. *Human molecular genetics* **19**, 573 (Feb 15, 2010).
422. R. J. Ferrante *et al.*, Chemotherapy for the brain: the antitumor antibiotic mithramycin prolongs survival in a mouse model of Huntington's disease. *J Neurosci* **24**, 10335 (Nov 17, 2004).
423. H. Ryu *et al.*, ESET/SETDB1 gene expression and histone H3 (K9) trimethylation in Huntington's disease. *Proc Natl Acad Sci U S A* **103**, 19176 (Dec 12, 2006).
424. C. Zuccato *et al.*, Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet* **35**, 76 (Sep, 2003).
425. A. M. Lepagnol-Bestel *et al.*, DYRK1A interacts with the REST/NRSF-SWI/SNF chromatin remodelling complex to deregulate gene clusters involved in the neuronal phenotypic traits of Down syndrome. *Human molecular genetics* **18**, 1405 (Apr 15, 2009).
426. P. Mulligan *et al.*, CDYL bridges REST and histone methyltransferases for gene repression and suppression of cellular transformation. *Mol Cell* **32**, 718 (Dec 5, 2008).
427. C. Zuccato *et al.*, Widespread disruption of repressor element-1 silencing transcription factor/neuron-restrictive silencer factor occupancy at its target genes in Huntington's disease. *J Neurosci* **27**, 6972 (Jun 27, 2007).
428. M. O. Kim *et al.*, Altered histone monoubiquitylation mediated by mutant huntingtin induces transcriptional dysregulation. *J Neurosci* **28**, 3947 (Apr 9, 2008).
429. A. P. Feinberg, R. A. Irizarry, Evolution in health and medicine Sackler colloquium: Stochastic epigenetic variation as a driving force of development, evolutionary adaptation, and disease. *Proc Natl Acad Sci U S A* **107**, 1757 (2010).
430. K. D. Hansen *et al.*, Increased methylation variation in epigenetic domains across cancer types. *Nat Genet* **43**, 768 (Aug, 2011).
431. M. J. Fazzari, J. M. Greally, Introduction to epigenomics and epigenome-wide analysis. *Methods Mol Biol* **620**, 243 (2010).
432. A. M. Brunner, R. Y. Tweedie-Cullen, I. M. Mansuy, Epigenetic modifications of the neuroproteome. *Proteomics* **14**, 201100672 (Jun 14, 2012).
433. C. H. Arrowsmith, C. Bountra, P. V. Fish, K. Lee, M. Schapira, Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov* **11**, 384 (May, 2012).

## **8. Appendix 1: Early life stress promotes stress resilience in the offspring**

Katharina Gapp<sup>1</sup>, Francesca Manuella<sup>1</sup>, and Isabelle M. Mansuy<sup>1\*</sup>

<sup>1</sup> Brain Research Institute, University of Zürich and Swiss Federal Institute of Technology, Neuroscience Center Zürich

\* Corresponding author, Email: mansuy@hifo.uzh.ch

**To be submitted at Frontiers in Behavioral Neuroscience**

## **8.1. Abstract.**

Adverse environment during early life constitutes a major risk factor for the development of psychiatric disorders later in life. It does however not affect all individuals to the same extend and can trigger stress resilience (165). Early life stress induced psychopathologies can be passed on to the next generation, the underlying molecular mechanism involving epigenetic modifications in the germ line (22, 244). Whether such epigenetic inheritance expands to stress induced resilience is unknown. Using a mouse model we show that early life stress decreases anxiety levels in the stress exposed animals and their offspring, increases performance in a stressful active avoidance task in the offspring while not affecting learning under non-stressful conditions. Further we observe upregulated levels of Glucocorticoidreceptors in the exposed animals and their progeny, which are associated with decreased DNA methylation at a transcription factor binding side in the promoter region of this receptor gene in the hippocampus. Environmental enrichment after early life stress exposure counteracts the inheritance and reverses the early life stress induced behavioral and gene expression alterations in these animals.

## **8.2. Introduction:**

Effects of stress are mediated by the hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis enables the organism to adapt to acute or chronic stress (55). Such adaption involves activation of corticoid receptors such as the low affinity Glucocorticoid (GR) receptor and the high affinity Mineralocorticoid receptor (MR). The GR is crucial for a rapid shut down of stress responses (55). Being subject to epigenetic regulation (11) it plays an important role in long-term adaptive processes in response to early chronic stress (245, 246).

Besides increasing the risk to develop psychiatric disorders later in life (152, 244, 247-249), early trauma has also been reported to induce physiological and psychological responses leading to stress resilience in humans and rodents (152, 250). Further, the manifestation of the effect of early life stress critically depends on the environment encountered later on (5). Importantly, maladaptive alterations triggered by early life stress are now known to be heritable via epigenetic mechanisms (22, 244).

The intriguing possibility that early life stress triggered resilience might also be inherited across generations has not been addressed so far. Here we used a previously in our laboratory established mouse model of early trauma to study the potential transfer of stress induced resilience into the offspring and explored a potential underlying molecular mechanism involving epigenetic regulation of the (GR).

## **8.3. Materials and Methods**

### **8.3.1. Animals**

C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature- and humidity-controlled facility. Cages housing 3-5 mice were cleaned weekly, and water and food were provided ad libitum. Animals were assigned to each type of housing (standard, SH or enriched, EE) randomly upon weaning, and animals were housed within treatment (control, CO or unpredictable maternal separation with maternal stress, MSUS). All experimental manipulations were performed during the animals' active cycle and all experiments were performed in accordance with guidelines and regulations of the cantonal veterinary office, Zurich.

### **8.3.2. MSUS treatment and breeding**

For unpredictable maternal separation with maternal stress (MSUS), dams and litters were subjected to daily 3-hr proximal separation from PND1 to PND14. During separation, mothers and pups were placed in separate clean cages with bedding, and

dams cages contained food and water. Litters and dams were placed such that they had visual and olfactory contact. The timing of separation was unpredictable, but always occurred during the dark cycle. Maternal stress consisted of either 20-min restraint in a Plexiglas tube or 5-min forced swim in cold water (18°C) applied unpredictably and randomly during separation. Both MSUS and control dams and litters had their cages changed on PND1, PND7, PND14, and PND21, during which the pups were also weighed. Outside of this manipulation, control dams and litters were left undisturbed. Only dams giving birth less than a week apart of each other were used. Litters with less than 4 pups were excluded from the experiment.

### **8.3.3. Housing conditions**

Control and MSUS mice in standard housing (SH) were housed in groups of 3-4 animals per cage in Nalgene cages (32x17x14 cm) containing wood chip bedding and a small carton house. Control and MSUS mice (males) subjected to environmental enrichment (EE) were placed in social groups (n=12) in an enriched cage immediately after weaning until adulthood (Marlau©, Viewpoint). Behavioral testing was carried out after EE. The enriched cage consisted in a large box with two levels (55x36x19cm lower and 55x36x11cm upper floor). The bottom level was separated into two compartments, one containing food pellets, and the other with access to water and containing running wheels, and a covered/protected area. The top level consisted of a maze (35x36x11cm) whose shape and configuration were changed three times per week with a total of 12 different options. The box was organized such that animals had to go through the maze to reach the food compartment.

### **8.3.4. Behavioral Testing**

Throughout all behavioral testing the experimenter was blind to treatment and tracking was performed manually as well as automatically (Viewpoint System). Testing started with the least aversive task, under indirect, dim red light when animals were 3 months old. The Elevated plus maze was used to assess anxiety-like behaviors. The forced swim test was used to assess depressive-like behaviors, and the open field was used to assess behavioral response to novel or aversive environments.

#### *Light/dark box test (LDBT)*

Mice were placed in the light compartment of a plastic box (40 x 42 x 26cm) split into two compartments by a divider. The light (aversive) compartment comprises 2/3 of the area, has white walls and is brightly lit by an overhead lightbulb. The neighbouring dark (safe) compartment represents 1/3 of the surface area of the box, has black walls and is covered with a black lid. An opening in the divider (5x5cm) allows the mouse to move freely from one compartment to the other. Then, the animal was allowed to freely explore the box for 10 mins, during which the time spent in the light and dark compartments, and the latency to enter the dark compartment were measured manually.

#### *Active Avoidance*

Four identical operant conditioning chambers (159x165x175mm) with stainless steel grid floors (TSE Systems, Germany) were housed in sound-insulated cubicles. Each chamber was equipped with a shock grid floor, a nose-poke response unit (2-cm diameter) fitted with a photocell sensor and a yellow cue light in the hole. An additional green cue light was located on the wall on top of the nose-poke module. A house light (2.8 W) was located on the ceiling, which was on throughout each testing session. On the left side of one wall, a nose-poke module was situated, which was also fitted with a photocell-sensor.

Habituation. Mice were habituated to the chambers for 30 minutes, during which house light was on and the nose-poke module was closed.

Procedure. Two days after habituation animals were tested on the active avoidance schedule for 30 minutes. During this schedule animals underwent 60 escape trials, with shocks of 0.3 mA intensity delivered through the grid floor every 30 seconds. The shocks had a maximum duration of 10 seconds, but were terminated immediately if the animal emitted a nose-poking behavior. If the animal did not exhibit the nose-poking response, the shock was terminated automatically after 10 seconds. In all phases for all animals, the light cycle was as follows: the house light was always on, the green light was on during non-shock periods, and the red light was on during shock periods. The sensor located in the nose poke hole recorded the time of occurrence of each nose poke at a resolution of one sec. Latency to escape and number of escapes were measured in blocks of 5 trials.

#### *Fixed ratio (FR) paradigm*

One day before start of the experiment, animals were changed to restricted water access conditions. Body mass of animals was checked weekly. Mice were tested Monday to Friday and had free water access after finishing testing for one hour every day and on Fridays until 9:00 or 11:00 am on Sundays.

**Apparatus.** Four identical operant conditioning chambers (159x165x175mm) with stainless steel grid floors (TSE Systems, Germany) were housed in sound-insulated cubicles. Each chamber was equipped with a nose-poke response unit (2-cm diameter) fitted with a photocell sensor and a yellow cue light in the hole. An additional green cue light was located on the wall on top of the nose-poke module. A house light (2.8 W) was located on the ceiling, which was on throughout each testing session. In the middle of one wall, a liquid dispenser was situated left to the nose-poke module, which was also fitted with a photocell-sensor.

Mice were habituated to the chambers throughout one session lasting 30 minutes, during which 10 ul of water were delivered every 30 seconds to the liquid dipper.

For the following 12 days were trained on a continuous fixed ratio (FR) schedule to nose poke for the delivery of a liquid reinforcer during daily 30-min sessions. Activation of the nose poke led to delivery of 10ul water in the liquid dipper. Learning efficacy was calculated by the formula: total number of nose pokes/total number of collected drink rewards.

### **8.3.5. Quantitative RT-PCR**

DNasel-treated RNA isolated from hippocampus (Allprep RNA/DNA kit; Qiagen) was reverse transcribed (RT), using the SuperScript First-Strand Synthesis System II for RT-polymerase chain reaction (PCR; Invitrogen Carlsbad, California). Quantitative RT-PCR was performed in an ABI 7500 thermal cycler using TaqMan probes (Applied Biosystems Foster City, California; Mm01241596\_m1) as described previously(22).

### **8.3.6. Bisulfite pyrosequencing**

Genomic DNA from hippocampi and sperm was extracted with the Allprep-kit (Qiagen), according to the manufacturer's instructions. Bisulfite pyrosequencing was performed by EpigenDx as previously described (251, 252). Universally methylated and unmethylated DNA samples (Millipore Bioscience Research Regents) were used as controls.

### **8.3.7. Statistical analyses**

An independent two-way ANOVA with main factors of postnatal treatment (Control and MSUS) and housing (SH and EE) was used to analyze behavioral results of light dark test and q-PCR. Repeated measurements ANOVA with main factors of postnatal treatment (Control and MSUS) and housing (SH and EE) and within factor of blocks of 5 escape trials was applied to examine the results of the AAT. For analysis of FR 1 paradigm repeated measurements ANOVA with main factor of treatment and within factor of testing day was used. Results of q-RT PCR containing only two groups were analyzed using two tailed student's t-tests. All data analyzed matched the requirements for parametric statistical tests, if variances were not homogeneous between groups an adjusted p-value, t-value and degrees of freedom were reported. Significance was set at  $p<0.05$  for all tests. Error bars represent standard error of the mean (SEM).

## **8.4. Results**

### **8.4.1. Stress sensitivity in F2 males**

To evaluate whether the MSUS treatment triggers resilience in the mice exposed to stress F2 males were tested for stress sensitivity on the Light dark box test (LDBT), which assesses an animals coping behavior with a brightly lit field.

Statistical examination revealed a significant group effect of housing for the time spent in the bright compartment ( $F(1,99)=5,061$ ,  $p=0,027$ ) (Figure1A), which could be biased by an interaction between housing and treatment ( $F(1,99)=98,689$ ,  $p=0,004$ ) (Figure1A). There was a significant group effect of treatment on the latency to enter the dark compartment ( $F(1,99)=5,994$ ,  $p=0,016$ ) (Figure1B) which again could be biased by a significant interaction between treatment and housing for the latency to enter the dark compartment  $F(1,59)=4,708$ ,  $p=0,032$ ) (Figure1B). These results indicate a potential bidirectional effect of EE on anxiety depending on the life history (MSUS or control).

Further, analysis using T-tests showed a significant difference of the time spent in the bright compartment between the offspring of standard housed MSUS and control animals ( $t(42,675) = -3,353$   $p = 0.002$ ) (Figure3b), which did not persist between the offspring of MSUS exposed to EE and standard housed controlsF2 ( $t(49) = 1,631$ ,  $p <0,05$ ) (Figure1b), nor between the offspring of MSUS and controls exposed to EE ( $t(20.85)=1.94$ ,  $p=0.07$ ) indicating that exposure to EE of the fathers is sufficient to reverse MSUS induced decreased anxiety levels. Analysis of the latency to first enter

the dark compartment revealed no significant change between the offspring of standard housed MSUS and control animals ( $t(70)=0.31$ ,  $p=0.76$ ) (Figure 3b), but a significant increase in the offspring of control animals exposed to EE when compared to the offspring of MSUS ( $t(17.89)=2.33$ ,  $p=0.03$ ) exposed to EE.

#### **8.4.2. Stress sensitivity in F1 animals**

To examine whether animals that were directly exposed to stress already displayed a decreased stress sensitivity behavior that can be reversed by EE we tested F1 males on the LDBT.

Statistical examination of stress sensitivity using the light dark box test (LDBT) showed a significant group effect of housing ( $F(1,47)=57,813$ ,  $p=0,000$ ) (Supp. Figure 1A) and a significant interaction ( $F(1,47)=50,212$ ,  $p=0,000$ ) for the time spent in the light compartment (Figure 1E). There was no significant effect for the latency to first enter the dark compartment (Figure 1F).

Post hoc two tailed T-tests revealed a significant increase in the time spent in the bright compartment in COSH versus MSSH animals ( $t(35) = -2,138 p = 0.04$ ) (Figure 1E), which was even further increased between COSH and MSEE animals ( $t(24) = 4,689 p = 0.002$ ) (Figure 1E). Controls exposed to EE further showed increased time spent in the bright compartment when compared to standard housed controls ( $t(15,018) = -6,027 p = 0.00$ ) (Figure 1E) but no change in neither this measure ( $t(15)=-0.78$ ,  $p=0.45$ ) nor the latency to first enter the dark ( $t(16)=-0.38$ ,  $p=0.97$ ) when compared to MSUS exposed to EE.

#### **8.4.3. Active avoidance learning in F2 animals**

To further test whether the observed behavioral amelioration in F2 MSUS males and their reversal by EE in the parent generation holds up in a truly stressful paradigm we examined resilience in an active avoidance task (AVT).

Repeated measurement ANOVA with treatment and housing as between subject factors and time as a within subject factor showed a significant effect of treatment on the latency to escape ( $F(1,53)=4,722$ ,  $p=0,034$ ) (Figure 1C) and the number of escapes ( $F(1,51)=7,216$ ,  $p=0,01$ ) (Figure 1D). Further, we observed a tendency for an interaction between treatment and housing for the latency to escape ( $F(1,53)=3,074$ ,  $p=0,085$ ) (Figure 1C) and the number of escapes ( $F(1,53)=3,412$ ,  $p=0,071$ ) (Figure 1D). These findings indicate a strong effect of treatment independent of EE, and a

differential effect of EE on the active avoidance behavior depending on the history of the fathers.

Post hoc analysis by repeated measurement ANOVA with either treatment or housing as between subject factors and time again as within subject factor revealed a significant difference between the offspring of standard housed MSUS and the offspring of standard housed control animals ( $F(1,26)=7,736$ ,  $p=0,01$ ) and between the offspring of standard housed MSUS and the offspring of MSUS animals exposed to EE ( $F(1,24)=16,095$ ,  $p=0,001$ ) for the latency to escape (Figure 1C), suggesting increased levels of stress resilience in the offspring of standard housed MSUS animals which can be effectively counteracted by EE. There was no significant difference in the latency to escape between the offspring of MSUS animals exposed to EE and the offspring of standard housed control animals ( $F(1,26)=1,218$ ,  $p=0,28$ ) (Figure 1C), nor between the offspring of MSUS mice exposed to EE and the offspring of control animals exposed to EE ( $F(1,27)=0,088$ ,  $p=0,769$ ) (Figure 1C), nor between the offspring of standard housed control animals and the offspring of control animals exposed to EE ( $F(1,29)=0,463$ ,  $p=0,0502$ ) (Figure 1C). These findings further support the potential of EE to selectively counteract increased resilience, without affecting animals raised under normal conditions. Post hoc analysis of another variable assessed in this test, the number of escapes, also revealed a significant difference between the offspring of standard housed MSUS and the offspring of standard housed control animals ( $F(1, 23)=8.501$ ,  $p=0.01$ ) and between the offspring of standard housed MSUS and the offspring of MSUS animals exposed to EE ( $F(1,22)=16.43$ ,  $p=0.00$ ) for the number of escapes (Figure 1). There was no significant difference in the latency to escape between the offspring of MSUS animals exposed to EE and the offspring of standard housed control animals  $F(1,29)=.59$ ,  $p=0.45$  (Figure 1D), nor between the offspring of MSUS mice exposed to EE and the offspring of control animals exposed to EE ( $F(1,28)=.42$ ,  $p=.52$ ) (Figure 1D), nor between the offspring of standard housed control animals and the offspring of control animals exposed to EE ( $F(1,29)=.59$ ,  $p=0.45$ ) (Figure 1D). These findings corroborate the results on the latency to escape and thereby support the potential of EE to selectively counteract increased resilience, without affecting animals raised under normal conditions.

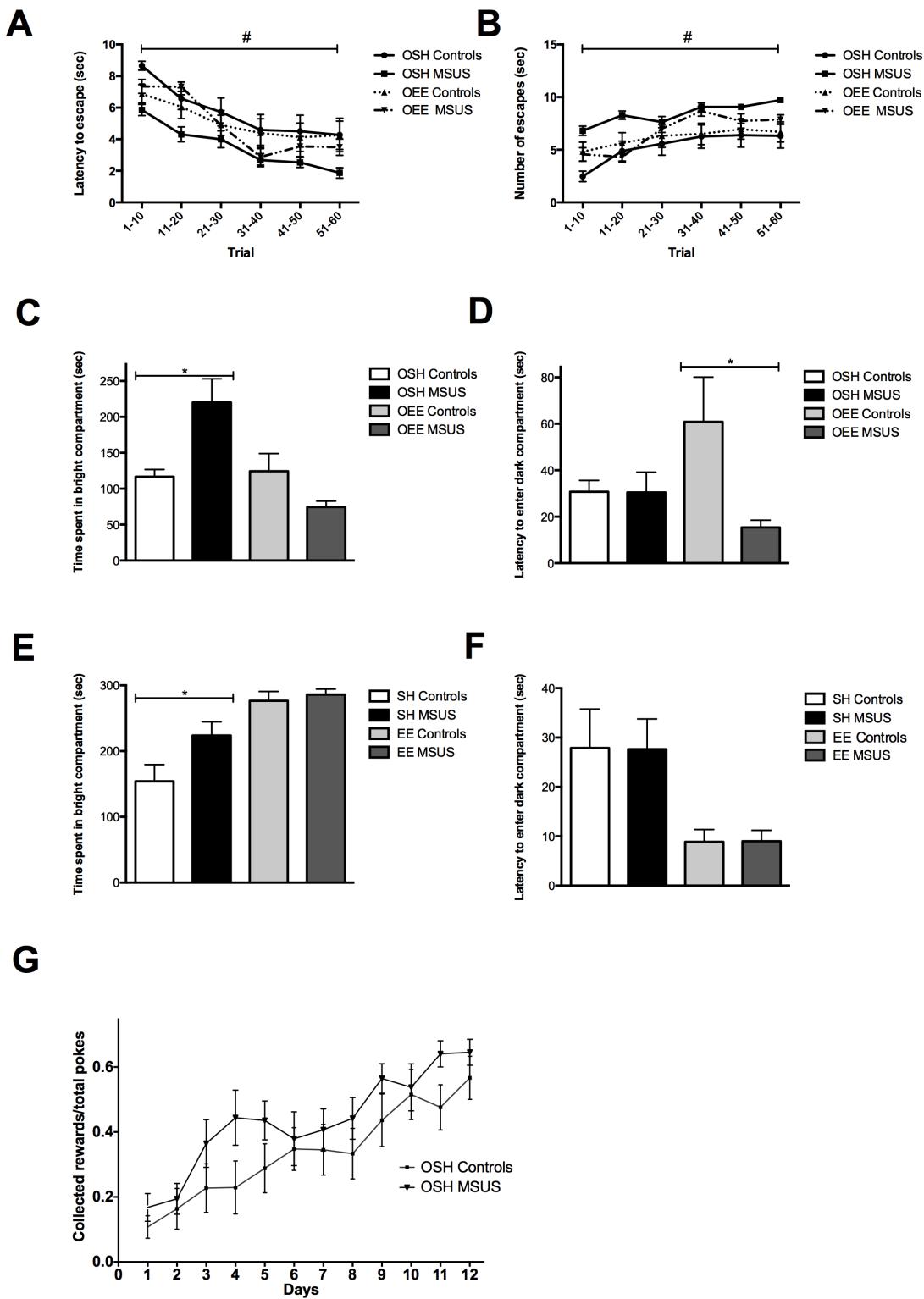
#### **8.4.4. Reward based learning in F2 animals**

To examine whether the observed effects of early life stress on F2 MSUS animals were specific to a resilience-like learning performance under stressful conditions we

performed a reward based learning paradigm, the FR1 schedule. Repeated measurements ANOVA revealed no effect of treatment on the learning efficacy in this learning task ( $F(1,26)=1,33$   $p=0,26$ ) (Figure 1G), disproving a generic impact of early life stress on learning abilities of the offspring.

#### **8.4.5. GR expression**

To investigate molecular mechanisms underlying the resilience and its reversal we studied Glucocorticoid receptor (GR) expression in the hippocampus, one of the key brain structures implicated in the negative regulation of stress responses(249). Univariate analysis of hippocampal gene expression in the offspring of MSUS animals either exposed to standard housing or enrichment revealed a significant group effect of treatment  $F(1,21)=7,739$ . Between the offspring of standard housed control and MSUS animals we observed a significant increase in GR expression ( $t(11)=-2,7$ ,  $p=0,021$ ), which did persist between the offspring of standard housed control and environmentally enriched animals ( $t(9,5)=-2,23$ ,  $p=0,05$ ), but did disappear between the offspring of control and MSUS animals exposed to EE ( $t(12)=-.84$ ,  $p=0,415$ ), indicating a reversal of the effect of MSUS on GR expression. F1 hippocampus also showed increased GR gene expression in standard housed controls as compared to MSUS (Figure 2B).



**Figure 1: Resilient behavior in the mice exposed to MSUS and their offspring and reversal by environmental enrichment.** MSUS induced significant differences in the offspring (A) in the latency to escape a shock, (B) the total number of shocks on active avoidance task and (C) the time spent in the bright compartment (D) the latency to first enter the bright compartment of the light dark box. (A-D) These changes were corrected by exposure of the fathers to EE. Animals directly exposed to MSUS show

alterations (E) in the time spent in the bright compartment (F) independent of the latency to first enter the bright compartment of the light dark box. (E,F) Environmental enrichment reversed the effect of MSUS. (G) The offspring of MSUS animals did not have altered performance on a reward based learning task. OSH offspring of standard housed males, OEE offspring of environmentally enriched males, SH standard housed, EE environmentally enriched. Data are presented as mean  $\pm$  SEM, n=7-9 litters/group, \*p<0.05 T-test comparison, # p<0.05 repeated measurements Anova comparison of offspring of SH.

#### **8.4.6. GR promoter DNA methylation**

Because the GR is known to be regulated by DNA methylation depending on the environment we next examined whether DNA methylation is altered by MSUS at the GR gene. Quantitative bisulfite pyrosequencing analyses of the untranslated exon1-7 region upstream of the transcription start site that contains transcription factor binding sites and is therefore important for transcriptional regulation(253) revealed a significant group effect of treatment at CpG3 (CpG3: F(1,61)=7.94, p=0,01) and a tendency for a group effect of housing at the same CpG side (F(1,23,31)=3.03, p=0,09) in F2 MSUS hippocampus (Figure 2C). Furthermore CpG4 showed a tendency for an interaction between housing and treatment (F(1,43,9)=3.97, p=0,05). Follow up T-tests revealed a significant decrease at CpG3 ( $t(26)=2.33$ , p=0,03) and CpG7 ( $t(26)=2.19$ , p=0,04) in the offspring of standard housed MSUS animals when compared to the offspring of standard housed controls, which did persist between the offspring of standard housed control and environmentally enriched MSUS animals at CpG3 ( $t(17)=-3.071$ , p=0.007), but not at CpG7 ( $t(4.354)=0.546$ , p=0.612). Further the differences were abolished between the offspring of controls and MSUS mice exposed to enrichment (CpG3:  $t(10)=2.17$ , p=0,06; CpG8:  $t(10)=-0.97$ . p=0,36), indicating a causal relation to altered GR expression. A decrease in methylation at CpG3 was also apparent in F1 MSUS hippocampus (CpG2:  $t(9)=3.43$ , p=0,007; CpG3: $t(9)=2.31$ , p=0,046 (Figure 2D).

Previous studies suggested DNA methylation in the germ line to account for the transgenerational inheritance of the effect of early life stress on gene expression (22). Hence we analyzed DNA methylation in sperm F1 cells and found a decrease in DNA methylation at the same side ( $t(8)=4.26$ , p=0,003) (Figure 2E), suggesting a mechanistic involvement in the epigenetic inheritance of the effect of MSUS.

## 8.5. Discussion

Resilience is a form of active coping with a stressful, challenging environment. It involves adaptation of the neuroendocrine system (254), but the specific underlying mechanisms are not fully elucidated yet. Many rodent studies report on animals that successfully adapt to chronic early life stress in form of maternal separation by altering the HPA axis (58, 152). During such process hippocampal GR plays a central role in negatively regulating stress responses (55, 255). Our lab has developed a model of unpredictable maternal separation that causes behavioral depressive like alterations associated with molecular alterations in the HPA axis. Importantly previous studies on this model have shown that these changes are persistent up to the third generations, independent of the maternal care in the offspring (22, 153). Such forms of intergenerational inheritance extend to other manipulations such as cocaine resistance(25), or obesity, endocrine and reproductive dysfunction caused by gestational exposure to endocrine disruptors (237) in rodents or metabolic alterations nutritional environment throughout life or during gestation in rodents and humans (30, 32).

In the current study we found that dependent on the testing environment (stressful versus non-stressful) the MSUS animals show stress resilience associated with altered epigenetic regulation of hippocampal GR that strikingly persists to the offspring and is associated with altered DNA methylation of the GR in the sperm. Reversal of the resilient behavior of the offspring by environmental enrichment in the stress-exposed generation was associated with rescue of epigenetic regulation of the GR.

We show that early life stress induces increased time spent in the bright compartment of the LDB as compared to the controls. This effect is not due to a difference in latency to enter the dark compartment. These data strongly support the concept of stress resilience (165) and the mismatch model (54), by showing ameliorated responses in a stressful environment, the LDBT, as compared to results of previous studies on MSUS animals showing impairments in less stressful paradigms such as the open field test (22). They corroborate previous findings by Roman et al. showing stress resilience after prolonged maternal separation combined with maternal stress in Wistar rats (256).

The difference between MSUS and controls does not persist between MSUS and control animals exposed to EE, indicating an anticipated correcting effect by EE (257).

The offspring of MSUS animals also spends more time in the bright compartment when compared to offspring of controls, again independent of the latency to enter the dark compartment. There is no difference however between the

offspring of the MSUS and control animals exposed to enrichment, indicating a transgenerational resilience induced by early life stress which can be reversed by exposure to EE of the parents.

When examining learning behavior under aversive environmental conditions in the AAT the offspring of MSUS animals had a clearly improved performance, reflected by a shorter latency to escape shocks and a higher number of escapes as compared to Controls. The offspring of enriched MSUS animals however was indistinguishable from that of enriched Control animals, indicating again a reversal by EE.

In comparison to this aversive learning paradigm we anticipated a reward based learning to be unaffected by MSUS of the parent generation. The number of pokes, each rewarded with a water droplet, of MSUS offspring was indistinguishable from that of control offspring, confirming the specificity of beneficial effects on performance to an aversive environment.

Importantly, our data on ameliorated responses to the LDBT and increased performance on an aversive learning paradigm the AAT in F2 males, while leaving non-stressful reward-based learning paradigms unaffected show for the first time that resilience is maintained in the second generation.

When studying the potential underlying molecular mechanisms we found that GR receptor expression is significantly increased in MSUS males, indicating adaption that could lead to a quicker shut down of the stress response (249). This increase was accompanied by a decrease in DNA methylation at exon1-7, which has previously been shown to be important for transcriptional regulation of hippocampal GR sensitive to levels of maternal care (11), strongly suggesting a causal relationship with the effect on gene expression. Strikingly, the effect was maintained in the offspring of these animals, being likely mediated across generations by the altered DNA methylation at the same locus in the germ line.

The differences in gene expression and methylation did not persist between the offspring of MSUS and control animals exposed to EE, corroborating the reversal effect of EE in the behavior.

Certainly, it is not possible to conclude from our data unambiguously that GR is the only contributor to the observed behavioral resilience in stressful environment, but as the reversal of the molecular phenotype is accompanied by a correcting effect on the behavior and previous studies identified GR as a crucial player in active avoidance (258) we can be confident that GR plays a key role in the studied behavioral effects.

Although, showing a reversal of the molecular and behavioral alterations our study does not determine whether the EE employs the same pathways as the early life stress. Findings from a study, that dissected the EE responsive time window in

combination with chronic restraint stress in adulthood, support that EE and chronic stress act on similar mechanisms within the hippocampus (257).

However, early life stress might recruit other molecular players than EE that subsequently trigger a reversed epigenetic regulation of the GR gene. Hence, whether our study involves a mechanistic reversal remains to be elucidated.

Intriguingly, the exposure to EE of MSUS males seems to have a negative impact on performance on their offspring as compared the offspring of controls exposed to EE. However, if one compares their learning to the offspring of standard housed controls performance is rather increased, albeit not significantly, further supporting the mismatch hypothesis (54).

Overall, our data extend the classical view of environmentally triggered resilience to a transgenerational priming mechanism for aversive environments.

## **8.6. Acknowledgements**

Our laboratory is supported by the University of Zürich, the Swiss Federal Institute of Technology, the Swiss National Science Foundation, the National Center for Competence in Research “Neural Plasticity and Repair,” the Human Frontier Science Program, the Borderline Personality Disorder Research Foundation, Roche, the European Molecular Biology Organization Young Investigator Program, and the Novartis Research Foundation. We thank Megan Frugoli for help with the behavioral tests, Tamara Franklin for help in the preparation of samples for pyrosequencing and Johannes Bohacek, Carmen Sandi and Melly Oitzl for critical discussion of the results.

## **8.7. Authors` contribution**

Katharina Gapp and Francesca Manuella applied the MSUS and EE paradigm and carried out the behavioral tests. Katharina Gapp prepared samples and carried out molecular experiments. Katharina Gapp and Isabelle Mansuy designed the study, interpreted the results and wrote the manuscript.

## **9. Appendix 2: Epigenetic regulation in neurodevelopment and neurodegenerative diseases**

Katharina Gapp, Bisrat T. Woldemichael, Johannes Bohacek and Isabelle M. Mansuy

*Brain Research Institute, Medical Faculty of the University of Zürich and Swiss Federal Institute of Technology, Neuroscience Center Zürich, Zürich, Switzerland.*

Corresponding author: IMM, mansuy@hifo.uzh.ch

### **Highlights:**

- This review describes the involvement of epigenetic processes in neurodevelopment and across life, and their contribution to neurodevelopmental and neurodegenerative diseases.
- Animal models used to study epigenetic alterations associated with these diseases and knowledge gained into their potential contribution are discussed.
- The potential of epigenetic drugs for intervention is also discussed.

*"Between genotype and phenotype, and connecting them to each other, there lies a whole complex of developmental processes."* Waddington, 1942

**Published in Neuroscience, 2012 (in press)**

## **9.1. Abstract**

From fertilization throughout development and until death, cellular programs in individual cells are dynamically regulated to fulfill multiple functions ranging from cell lineage specification to adaptation to internal and external stimuli. Such regulation is of major importance in brain cells, because the brain continues to develop long after birth and incorporates information from the environment across life. When compromised, these regulatory mechanisms can have detrimental consequences on neurodevelopment and lead to severe brain pathologies and neurodegenerative diseases in the adult individual. Elucidating these processes is essential to better understand their implication in disease etiology. Because they are strongly influenced by environmental factors, they have been postulated to depend on epigenetic mechanisms. This review describes recent studies that have identified epigenetic dysfunctions in the pathophysiology of several neurodevelopmental and neurodegenerative diseases. It discusses currently known pathways and molecular targets implicated in pathologies including imprinting disorders, Rett syndrome, and Alzheimer, Parkinson and Huntington disease, and their relevance to these diseases.

**Keywords:** neurodevelopment, epigenetics, imprinting disorders, Rett syndrome, Alzheimer's disease, Parkinson's disease, Huntington's disease,

## **9.2. Introduction**

The question of how cells in an organism develop into distinct types and fulfill different functions despite carrying the same genetic information has intrigued biologists for decades. Further, how environmental conditions can allow cellular systems to acquire specific features such as resistance to stress, and keep these features throughout life is also still poorly understood (259, 260). In an attempt to explain these phenomena, Conrad Waddington (1942) introduced the concept of epigenetics, and proposed the idea that environmental factors can modify a fixed genotype, alter developmental processes and thereby confer specific properties to cells. Epigenetics refers to changes in the functions of the genome that occur without any alteration in the DNA sequence itself (261). Although initially restricted to heritable changes in cellular features, it also now includes non-heritable changes. The mechanisms underlying epigenetic processes have been extensively studied in the past decades. Their major functions are thought to be to dynamically regulate gene activity in response to environmental events, and provide functional epigenomic signatures that can persist. While they operate in all cells and tissues, they are particularly important for the nervous system.

This review provides a brief synopsis of the processes of epigenetic regulation in the brain in health and disease across life. It first focuses on epigenetic mechanisms during early brain development, adult neurogenesis and aging, then discusses their implication in the pathophysiology of neurodegenerative disorders. It also discusses the possibility of novel therapeutic approaches based on the manipulation of epigenetic processes.

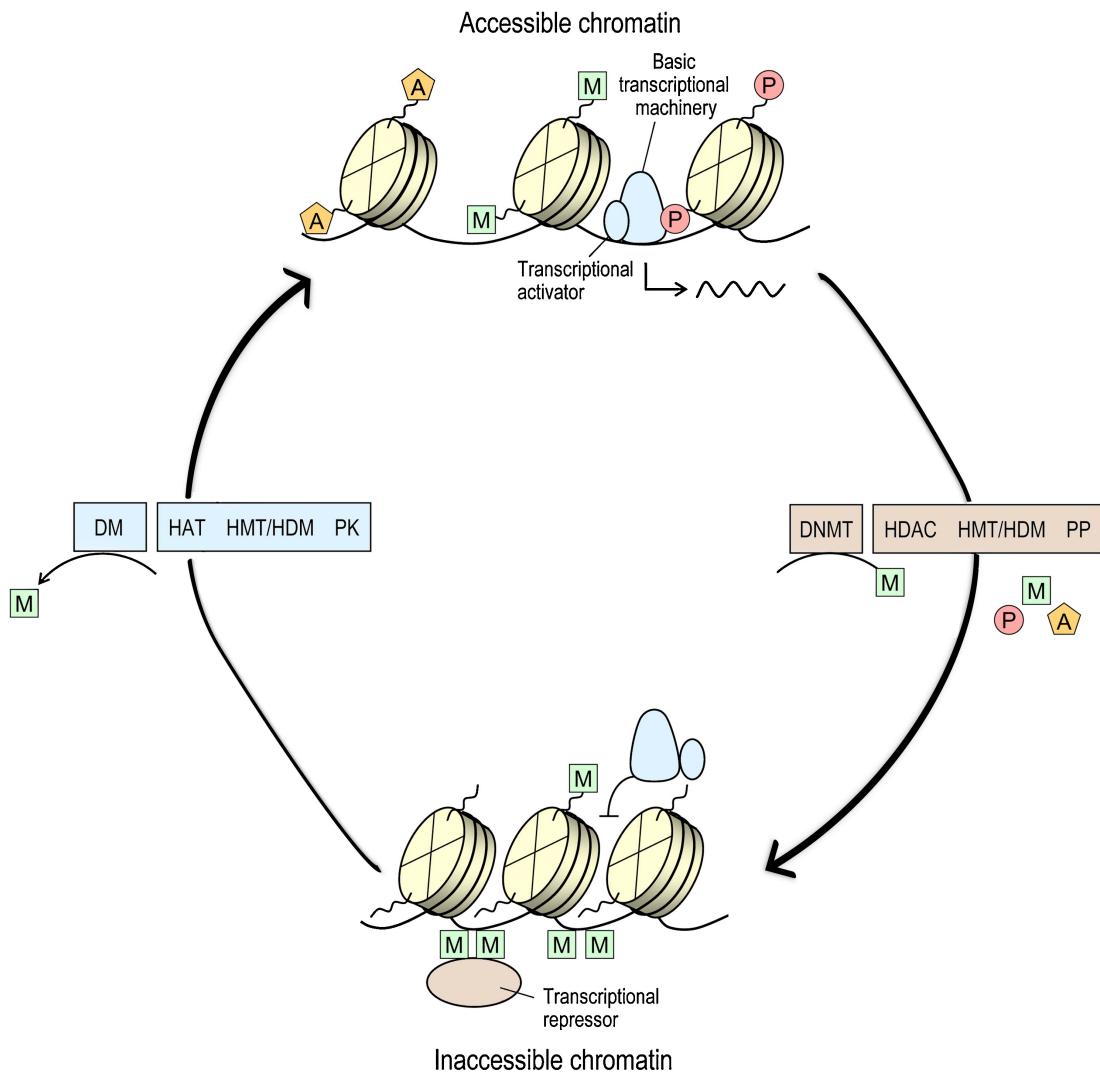
## **9.3. Epigenetics in brain development and aging**

### **9.3.1. DNA methylation and histone posttranslational modifications**

Chromatin remodeling is an essential feature of the DNA required for the regulation of gene expression. It is a complex ensemble of mechanisms mediated by epigenetic and structural processes in particular, DNA methylation and histone posttranslational modifications (PTMs). DNA methylation is one of the best known epigenetic modes of regulation by which a methyl group is added to the 5' carbon of cytosine in dinucleotide CpG sequences. It is induced by *de novo* DNA methyltransferases

(DNMTs) such as DNMT3a and b, and maintained by DNMT1 (262). CpGs are mainly found in clusters known as CpG islands (CGI), often located in the promoter region of genes and mostly hypomethylated when compared to CGI shores (sequences spanning 2kb up- and downstream of each CGI) and gene bodies (263). Increased methylation of CGI has a strong impact on gene transcription, and generally leads to gene silencing (264, 265). Silencing can result from negative DNA charges that create conditions preventing chromatin opening, and from the recruitment of transcriptional repressors (60). For instance, DNA methyl-binding domain proteins (MBD1-4) and methyl-CpG binding protein 2 (MeCP2) are recruited to methylated DNA, and engage histone deacetylases (HDACs) complexes (9, 266). But DNA methylation can also be associated with transcriptional activation, through mechanisms that remain unknown (267, 268).

PTM of histone proteins is another epigenetic mechanism that, together with DNA methylation, alters chromatin structure. PTMs are covalent modifications of specific residues on histones N- or C-terminus tail or core and include acetylation, phosphorylation, methylation (mono, bi or tri), ubiquitylation, sumoylation or crotonylation (269, 270). They are regulated by specific enzymes such as histone acetyl transferases (HATs), HDACs, protein kinases and phosphatases, histone methyl transferases (HMTs) and demethylases (HDMs), present in chromatin modifying complexes. Histone PTMs can act in *cis* or *trans*, and strongly influence each other to form a dynamic histone code specific for each gene. By altering the net charge of nucleosomes, they modify DNA-histone interactions leading to structural changes, and transcriptional activation or silencing (7, 271). Histone PTMs can also recruit chromatin modifying enzymes and/or DNA binding factors. For example, methylation of lysine at position 4 of histone 3 (H3K4) can inhibit the binding of HDACs thereby favoring acetylation, while acetylation of H3K18 facilitates the recruitment of the HAT CREB binding protein (CBP) (7, 271). Other epigenetic mechanisms not considered in the present review include DNA hydroxymethylation and regulation of gene expression by small RNAs (272, 273).



**Figure 1: Schematic representation of epigenetic modifications that regulate chromatin state and gene transcription.** Epigenetic marks such as histone acetylation, phosphorylation, and methylation favor electrostatic repulsion between histone proteins, making the chromatin less condensed and more accessible to the transcriptional machinery (Top). In contrast, methylation of DNA and histones decreases electrostatic repulsion between histones proteins, leading to chromatin condensation and lower accessibility to the transcriptional machinery. The transition between these chromatin states is regulated by specific epigenetic marks (in general DNA demethylation, some histone PTMs) catalyzed by various enzymes. DM, DNA demethylase; DNMT, DNA methyl transferase; HAT, histone acetyl transferase; HDAC, histone deacetylase; HDM, histone demethylase; HMT, histone methyltransferase; A, Acetylation; M, methylation; P, phosphorylation; PK, protein kinase; PP, protein phosphatase; TX, transcription. Adapted from (274).

### **9.3.2. Neurodevelopment**

During development, the nervous system arises from the ectoderm, the outer layer of the embryo. This process is initiated by formation of the neural tube followed by successive steps of cell proliferation, migration, neural patterning, cell maturation and establishment of neuronal connectivity (275, 276). Neurons and glia arise from neural progenitor cells located in highly proliferative areas including the subventricular zone. Initially, precursor cells are mainly differentiated into neurons that migrate to developing cortical areas. Then, depending on environmental signals, precursor differentiation switches to a glial fate (277). These steps require temporally regulated waves of gene expression across key developmental stages and are, in part regulated by epigenetic mechanisms.

During early development, two major stages of epigenetic programming control the fate of toti- and pluripotent embryonic cells. A first stage involves DNA demethylation/remethylation and reprogramming of histone PTMs in somatic cells, and a second stage, the erasure and reestablishment of parental imprints (by DNA methylation) during germ cell development (278). Subsequent stages of development also depend on DNA methylation.

To prevent non-neuronal cells from differentiating into neurons, proneural genes are kept in an inactive state. This is in part achieved by DNA methylation of neuron-restrictive silencer element (NRSE) in the promoter region of genes such as sodium channel type II, BDNF or calbindin (279, 280). Neuronal commitment then requires de-repression and (re)activation of neuronal genes such as Sox2 via decreased DNA methylation (281). Neuronal specification of proliferating cells during early neocortical development is also associated with the silencing of astrocytic gene loci and suppression of astrogliogenesis by DNA methylation. This silencing is attenuated at later stages of neocortical development and results in the generation of astrocytes which correlates with suppression of neurogenesis (276). Demethylation and expression of the genes coding for the astrocytic markers glial fibrillary acidic protein (GFAP) and S100 $\beta$  during astrocytic maturation for instance, co-occur with methylation and down-regulation of neurogenic genes such as Neurogenin1 (282-285).

The dynamic and reversible changes in DNA methylation during embryogenesis also coincide with a tight regulation of chromatin modifying enzymes during development. DNMT1 is active in dividing neural progenitor cells and mature neurons where it

maintains DNA methylation (286, 287). It is also highly expressed in the embryonic CNS and in post-mitotic neurons in perinatal and adult CNS (286, 288). Studies in DNMT1-deficient cortical neurons have shown that a lack of DNMT1 alters neuronal excitability, increases neuronal cell death (289) and in NSCs, compromises astrocytic differentiation (285). Further to DNMT1, DNMT3a is highly expressed during neuronal maturation, and contributes to neurogenesis and synaptic plasticity in adult neurons (287, 290). DNMT3a-deficient embryonic stem cells (ESCs) have precocious glial differentiation and increased proliferation (291). DNMT3b is mostly expressed in early embryonic cells and neural progenitors (292, 293). Depletion of DNMT3b leads to a failure of neuronal differentiation *in vitro* (294). Such dynamic expression and active regulation suggest that DNMTs are essential regulators of brain development.

Like DNA methylation, histone PTMs are also temporally and spatially regulated. In pluripotent ESCs, key developmental genes carry repressive or activating histone marks, such as trimethylation of H3K27 and H3K4 respectively, which confer ESCs a bivalent state (276). During ESCs commitment to neural precursor cell fate, H3K27 methylation decreases following up-regulation of the HDM JmjD3 and down-regulation of the HMT enhancer of Zest homologue 2 (EZH2). This results in a loss of the bivalent state then in fate commitment (295-297). In parallel, histone deacetylation also represses the expression of genes regulating fate commitment in non-differentiating NSCs. For example, the expression of Mash-1, an important regulator of fate commitment in NSCs is suppressed by histone deacetylation. However, upon neuronal differentiation, HATs are recruited, histone acetylation is induced and Mash-1 is expressed. Other neuron-specific genes such as NeuroD and Cdkn1c are also activated following histone acetylation (282, 298). But at the same time however, decreased HDAC activity at proneural genes also favors neuronal fate commitment. Thus, blockade of deacetylation by the HDAC inhibitor valproic acid, promotes neurogenesis and induces the expression of genes that block oligodendrocyte progenitor maturation such as NeuroD (299, 300). Thus, both histone acetylation and deacetylation play a role in conferring neuronal cell identity.

Regulation of glial genes by epigenetic mechanisms also contributes to the establishment of brain cell fate. During the switch to glial fate, GFAP expression in astrocytes is facilitated by recruitment of the HAT CBP to STAT3 binding element in the GFAP promoter (283). Oligodendrocyte fate commitment is accompanied by decreased histone deacetylation at neuronal genes such as Sox2 (301) or at transcriptional repressors of oligodendrocytic differentiation such as Sox11 (302). Further oligodendrocytes are also repressed by increased H3K9 dimethylation (303).

### **9.3.3. Adult neurogenesis**

Although the mature brain was long thought to have only differentiated post-mitotic cells, it is now recognized to also have proliferating cells in several neurogenic regions. These cells arise from the ventricular zone and the peripheral subventricular zone, two neural stem cell niches that undergo neurogenesis throughout life, and are mostly found in the dentate gyrus (a part of the hippocampal formation). In these niches, newly generated cells differentiate into neurons and glia (304). The role of adult neurogenesis in brain functions remains unclear and somewhat controversial, but evidence has suggested that it is important for memory processes, and may be implicated in psychiatric and neurological disorders such as depression, addiction and epilepsy (305). Notably, adult neurogenesis is highly responsive to environmental factors. For instance, it can be positively or negatively influenced by diet, and such influence may provide a link between diet and mental health (reviewed in (306)). It can also be increased by physical exercise and enriched environment (307, 308), in particular in aged mice. Thus, physical exercise enhances hippocampal neurogenesis and learning in aging, suggesting a functional relevance of adult neurogenesis (309).

The influence of environmental factors on adult neurogenesis has been shown to depend on epigenetic processes. The proliferation and differentiation of newly born cells into neurons are associated with epigenetic plasticity, and involve crosstalks between epigenetic marks. Adult neurogenesis requires DNA de-methylation; it is blocked in the hippocampus when Gadd45b, an immediate early gene (IEG) encoding a protein with DNA demethylase activity, is knocked-out in mice (310). It also requires histone acetylation and the proliferation of adult neural progenitors is blocked by HDACs inhibitors such as valproic acid *in vivo*. Valproic acid also facilitates neuronal fate commitment of progenitor cells and neuronal differentiation in part through increased NeuroD expression (300). Further, adult neurogenesis is regulated by several chromatin remodeling components. This includes REST/NRSF, a factor necessary for gene expression in the CNS and which diminishes neurogenesis (311), the TrxG complex element MII1 which is required for neuronal differentiation in the adult subventricular zone (312, 313), and Bmi-1, a major component of the polycomb repressive complex (PRC1) (313). Finally, miRNAs also play an important role in adult neurogenesis. For instance, miR-137, a regulator of Ezh2, another component of PRC1, decreases neurogenesis in forebrain NSCs (314), while miR-184 inhibits

differentiation of hippocampal adult NSCs by binding and repressing Numbl, a signaling protein required for differentiation (315).

#### **9.3.4. Aging and age-associated cognitive decline**

Like other organs in the body, the brain ages, and aging is often associated with brain dysfunctions, cognitive decline and neurodegeneration. At the genomic level, cognitive aging is accompanied by alterations in transcriptional programs in brain cells. In human, many genes important for fundamental neuronal functions such as synaptic plasticity, transport of vesicles and mitochondrial functions are downregulated in the cerebral cortex after age 40 (316-319). Likewise in aged rodents, the expression of multiple plasticity, signaling, and neuroenergetics components is altered. This has been correlated with increased DNA methylation (320, 321), particularly at genes involved in DNA binding, morphogenesis, and transcriptional regulation (322, 323).

The importance of dynamic epigenetic regulation in learning and memory formation has been well documented (138, 324, 325) and increasing evidence suggests that epigenetic dysregulation underlies aging-associated memory impairment. DNA methylation is increased at the promoter and intra-genic regions of IEGs such as the activity-dependent cytoskeleton-associated protein (Arc), a gene necessary for memory processes, and is associated with reduced gene expression and memory deficits in aged mice (326, 327). Likewise, epigenetic dysregulation involving histone PTMs has been linked to altered expression of plasticity-related genes in old animals. While in young adult mice, several genes including Prkca, Shank3, and Gsk3a are up-regulated following contextual fear conditioning and fear memory formation, and correlate with H4K12 acetylation, they are not in aged mice (328). Restoring acetylation by the HDAC inhibitor SAHA before training reverses the memory deficits in aged mice. Similarly, chronic HDAC inhibitor treatment or environmental enrichment in aged mice subjected to neurodegeneration and with deficient associative memory and spatial learning, restores these functions (329). Notably, while many epigenetic marks are coupled with memory performance in cognitively fit young adult and aged rodents (138, 325), such association is lost in aged animals with impaired memory (330). These findings overall suggest that epigenetic dysregulation in aging has detrimental consequences on cognitive functions, and may underlie cognitive decline in the aging population.

## **9.4. CNS disorders with an epigenetic basis**

### **9.4.1. Neurodevelopmental disorders**

Behavioral abnormalities during early life are often associated with compromised CNS development and lead to neurodevelopmental disorders later in life. In many cases, these diseases are associated with epigenetic dysregulation.

#### **9.4.1.1. *Imprinting disorders***

Diploid organisms inherit two copies of each gene from their parents, a maternal and a paternal allele. While most genes are expressed from both alleles, some are preferentially expressed from either the maternal or the paternal allele. Such selection is known as genomic imprinting and affects about 100 genes in mammals. It is established in the parental germline and results in a non-Mendelian bias in gene expression. Mechanistically, imprinting depends on DNA methylation (331, 332), and involves imprint control regions in imprinted genes which are differentially methylated on the maternal and paternal allele. Monoallelic expression of imprinted genes makes these loci particularly vulnerable since a mutation or deregulation of the expressed allele can compromise expression that cannot be compensated for by the other allele and can lead to severe developmental defects such as is the case for Angelman, Prader-Willi (333, 334) and Beckmann Wiedemann syndrome (335).

Angelman and Prader Willi syndrome are neurodevelopmental disorders that have related mechanisms but distinct clinical manifestations. Angelman syndrome is characterized by severe intellectual disability, ataxia, jerky arm movements and seizures, while Prader Willi syndrome is characterized by mild mental retardation, hypogonadism, early childhood onset hyperphagia and morbid obesity. Both diseases result from alterations in the genomic imprinting cluster on chromosome 15 (15q11-15q13). In this cluster, expression of imprinted genes is controlled by differential DNA methylation at a bipartite imprinting center in the SNRPN gene (334). One element of this center silences some of the paternal alleles in the cluster, while the other silences maternal alleles within the same cluster. In Angelman's syndrome, the paternal allele of UBE3A (ubiquitin protein ligase E3A) is silenced in neurons, and the maternal allele is altered by a mutation, deletion, or epigenetic inactivation resulting in abolition of UBE3A expression (334, 336, 337). In contrast, Prader Willi syndrome results from a deregulation of the paternal copy of several genes within the imprinting cluster that in normal conditions, are silenced only in the maternal allele (338). These disorders

exemplify the importance and impact of a mutation or deletion of the expressed allele in an imprinted locus.

#### **9.4.1.2. Rett syndrome**

Rett syndrome is an X-linked neurological disorder with early onset (18 months) that affects one girl in 10,000-15,000 (339). It is associated with severe mental retardation and motor impairment such as ataxia, apraxia, and tremor (340). Mutations in the gene coding for the transcriptional repressor MBD MeCP2 leading to a non-functional protein have been identified as one of the causes of Rett syndrome (268, 341). The exact pathway by which MeCP2 deficiency leads to Rett syndrome is however still unclear. It is known that a tight regulation of MeCP2 expression is essential for proper neurodevelopment, and both MeCP2 over- and under-expression lead to neurodevelopmental defects (340, 342). Further, although Rett syndrome has been mainly associated with MeCP2 disruption in neurons (343), its alteration in glial cells and in the periphery has also been implicated (344, 345) (346). The pathology of Rett syndrome has been modeled in animal models by introducing specific deletions in the MeCP2 gene. Consistent with a primary role of MeCP2, most models have lower brain weight, smaller neurons, and reduced synaptic plasticity and display cognitive deficits and altered exploratory activity (343, 347, 348) (342, 349, 350). Several of these symptoms can be reversed by rescuing MeCP2 expression or overexpressing MeCP2 (342), confirming the direct implication of MeCP2 (351, 352).

Mechanistically, although MeCP2 can broadly bind to methylated DNA, it appears to have some specificity and associate to only a subset of genes, leading to selective (and not genome-wide) changes in gene expression (353, 354). Such selectivity is not well understood but is thought to involve PTMs of MeCP2 itself such as phosphorylation, that may confer binding specificity and alter chromatin compaction at the target gene (355). MeCP2 binding is associated with changes in the level of methylation at specific target genes such as genes involved in neuronal plasticity like BDNF. Synaptic activity induces MeCP2 phosphorylation, which causes its release from the BDNF promoter, promoter demethylation and induction of BDNF expression (356, 357). Further, MeCP2 phosphorylation also alters the expression of genes involved in development, dendritic growth and spine maturation (268, 358) (359). Disruption of MeCP2 phosphorylation alters synapse development and behavioral response (360). Further to interfering with DNA methylation, a deficiency in MeCP2 also affects histone acetylation and methylation. In non-pathological conditions,

MeCP2 at methylated DNA recruits HDAC1 (357, 361) which leads to H3 and H4 hypoacetylation, and activation of H3K9 methylating enzymes (362). Consistently, dysfunctional MeCP2 is associated with H3 and H4 hyperacetylation, and decreases gene silencing by H3K9 methylation, which results in increased gene transcription. Additionally, it increases H3K4 methylation and further promotes gene transcription (7). Consistently, mice carrying a truncated form of MeCP2 have hyperacetylated H3 at the promoter of IEGs (363). These findings suggest that Rett syndrome is associated with an overall lack of inhibition of gene transcription. Recently, miRNAs have also been proposed to contribute to the pathology of Rett syndrome. In a Rett mouse model, MeCP2 deficiency was associated with a dysregulation of miRNAs, and a severe disruption of miRNAs expression in the brain. One of these miRNAs for instance is miR146a, which expression is increased and is functionally associated with up-regulation of IL-1 receptor-associated kinase 1 (Irak1) (364). The ensemble of these findings suggests complex mechanisms for Rett syndrome that implicate multiple epigenetic processes.

#### **9.4.2. Neurodegenerative disorders**

Neurodegenerative disorders such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) disease affect a large population worldwide. Several genes have been proposed to cause these diseases, but none alone has been directly linked to the etiology and expression of the disorders. In identical twins, disease incidence is largely discordant, despite similar genetic identity, suggesting that environmental factors largely contribute. Epigenetic mechanisms are thus thought to mediate the interaction between genetic and environmental factors and underlie the emergence of such complex disorders (365-368). They may also provide a 'cellular memory' that maintains a pathological state across life and in some cases, even across generations (274, 369-371). The following section describes examples of neurodegenerative disorders associated with epigenetic dysregulation.

##### **9.4.2.1. *Alzheimer's disease (AD)***

AD is one of the most frequent neurodegenerative diseases leading to dementia and that affects about 6% of the population over 65 (Burns and Iliffe, 2009). At a cellular level, the disease is associated with a loss of neurons and altered synaptic connectivity. They are caused primarily by the accumulation of extracellular plaques of amyloid- $\beta$  ( $A\beta$ ) peptide produced from the altered processing of the amyloid precursor

protein (APP), and neurofibrillary tangles containing hyperphosphorylated tau protein (372, 373). AD can have an early (familial) or late-onset form, and the risk for both forms is increased by mutations (rare) in APP, presenilin (PS) and apolipoprotein E4 (ApoE4) genes (374-376).

Environmental factors such as exposure to metals, traumatic brain injury, and early life stress have been described as predisposing factors to the disease. These factors can induce a wide range of epigenetic alterations that likely contribute to the pathogenesis of AD (369, 377-379). AD patients and animal models have dysregulated DNA methylation, and altered expression of AD susceptibility genes. However, while global DNA hypomethylation has been observed in the entorhinal cortex of some AD patients (380), DNA hypermethylation was also detected at several loci such as PS1 and LINE-1 in the dorsolateral prefrontal cortex of other AD patients (322, 381-383). This suggests that DNA methylation is differentially affected in a region- and locus-specific manner. The mechanisms for altered DNA methylation are not known but may involve dysregulated DNMT activity. In a primate model of AD, DNMT1 activity was found to be decreased and APP mRNA expression is increased in the cortex (384). But A $\beta$  itself may also be linked to altered DNA methylation. Higher A $\beta$  concentration results in hypermethylation of neprilysin gene, that encodes a major A $\beta$ -degrading enzyme in the brain which is decreased in the hippocampus in AD patients (385-387).

Histone PTMs are also dysregulated in AD, and histone acetylation is overall increased in the AD brain. This was proposed to be due to APP C-terminal peptide (AICD), a product of APP cleavage that interacts with the HAT TIP60 to increase acetylation (388). The HAT CBP may also be implicated as its proteasomal degradation is blocked by PS1 mutations leading to increased acetylation and CREB-mediated gene expression (389). Consistently, overexpression of the HDAC SIRT1 produces neuroprotection in a mouse model of AD (390). However, several studies have recently shown that decreased histone acetylation can also be associated with AD. APP overexpression in cultured cortical neurons reduces overall H3 and H4 acetylation, and decreases CBP level (391). Moreover, loss-of-function mutations in PS1 and PS2 reduce the expression of CBP and CBP/CREB target genes such as c-fos and BDNF. These alterations are accompanied by impaired synaptic plasticity and memory formation (392). Thus, H4 acetylation is reduced in the hippocampus of APP/PS1 mice following contextual fear conditioning training. Treatment with HDAC inhibitors restores acetylation (H3K4), and rescues memory deficits and synaptic

connectivity alterations in this mouse model of AD (329, 393), consistent with the implication of protein acetylation.

The fact that multiple epigenetic mechanisms are dysregulated in AD makes it challenging to delineate the pathology of the disease and develop effective therapeutics. However, epigenetic drugs such as HDAC inhibitors hold promise and may be useful in some treatments. Manipulations reversing the global DNA hypomethylation observed in AD brains may therefore be envisaged (325) (see also (394, 395)).

#### **9.4.2.2. *Parkinson's disease (PD)***

PD is a common neurodegenerative disorder affecting up to 6 million people worldwide (396). It is characterized by tremor, deficient initiation and control of voluntary movements, muscular rigidity, and postural instability. In advanced stages of the disease, non-motoric emotional and cognitive deficits also appear (397). The disease's pathogenesis involves a selective loss of dopaminergic neurons in nigro-striatal pathways, which reduces dopamine input to the striatum and perturbs the circuitry for motor coordination (398).

A number of gene mutations, including in SNCA, parkin (PARK2) and PTEN-INDUCED KINASE PROTEIN 1 (PINK1) have been identified as risk factors for PD (399, 400). However, only a minority of PD cases can be attributed to a single genetic factor and thus, up to 90% of disease cases are sporadic, pointing to a prevalent contribution of environmental factors. Indeed, epigenetics has become a major focus in PD research in the past years (401-403) and several links between PD and epigenetic processes have been identified. Increased alpha-synuclein (SNCA) expression in dopaminergic neurons associated with sporadic PD correlates with SNCA hypomethylation in PD patients (404). But at the same time, decreased histone acetylation has been linked with increased SNCA level in blood cells in patients with a gene mutation in some familial PD cases (405). Interestingly, SNCA itself is known to associate with histones and inhibit their acetylation (406). Thus, mutations in SNCA could lead to increased histone acetylation at the unaffected SNCA allele and increase its expression. The effect of SNCA may be mediated through HDAC Sirt2 as Sirt2 siRNA treatment can rescue cells from SNCA-mediated toxicity *in vitro* (407).

Another link between epigenetic modifications and PD involves the tumor necrosis factor alpha (TNF-alpha), a key inflammatory cytokine. The level of TNF-alpha is increased in the CSF of patients with PD (408) and TNF-alpha overexpression in neuronal cells induces apoptosis. Cell death in the substantia nigra pars compacta (SNpc), suggests that dopamine neurons in this structure might be particularly sensitive to TNF-alpha toxicity. Indeed, DNA methylation at the TNF-alpha promoter is reduced in SNpc cells, and may underlie the increased susceptibility of dopaminergic neurons to inflammatory reactions mediated by TNF-alpha (409). The implication of histone PTMs has further been supported by findings in animal models of PD. Rodent and primate PD models based on the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) that induces dopamine depletion have been associated with reduced striatal H3K4me3 (410). Conversely, raising dopamine level by administration of the dopamine precursor levodopa partially ameliorates the symptoms and correlates with deacetylation of H4K5, K8, K12, K16 (410).

#### **9.4.2.3. *Huntington's disease (HD)***

HD is a dominant autosomal neurodegenerative disorder characterized by cognitive decline, changes in personality involving impulsivity, depression, and psychotic symptoms, but also by motor dysfunctions including chorea, rigidity and dystonia. The pathophysiology of HD results from a progressive loss of inhibitory GABAergic neurons in the putamen and the caudate nucleus, which are involved in the motor circuitry. The cause of the selective loss of GABAergic neurons is not well understood. Genetic studies have involved trinucleotide repeat expansion in the huntingtin (Htt) gene leading to the production of an expanded Htt protein that aggregates and forms intracellular inclusions. The number of repeats steadily increases across generations; it proportionally shortens the disease onset and exacerbates the severity of symptoms (411). While in other diseases with trinucleotide expansion, the intergenerational and somatic repeat instability and altered mRNA expression have been attributed to epigenetic dysregulation (412), such link is still unclear for HD. However, some evidence has accumulated to suggest that expanded Htt itself may interact with epigenetic co-regulators and alter their activity.

Animal models of HD expressing an expanded Htt gene have reduced H3 and H4 acetylation and increased methylation (413, 414). Interestingly, mutated Htt protein can enter the nucleus and bind to the acetyltransferase domain of the HATs CBP and p300/CBP-associated factor. Such binding inhibits HAT activity and interferes with CREB-mediated transcription (415). Consistently, HDAC inhibitors can alleviate motor

deficits and neuronal atrophy by increasing overall histone acetylation (416-420). A role for Htt in histone methylation has also been suggested. Full-length endogenous nuclear Htt can associate with polycomb repressive complex 2 (PCR2), a HMT that catalyzes the repressive mark H3K27me3, and increases its activity. Embryos lacking Htt have lower PRC2 activity and H3K27 methylation (421). In HD patients and R6/2 mouse models of HD, the repressive bi- and trimethyl H3K9, and the H3K9 HMT ESET are increased (422, 423). Htt also directly interacts with neuron-restrictive silencer factor (NRSF) and prevents its translocation to the nucleus (424). NRSF can associate with epigenetic regulators including the HDM SMCX, HDAC1, HDAC2, and the HMTs G9a and chromatin remodeling factor SWI/SNF (425, 426). Expanded Htt fails to sequester REST in the cytoplasm and thereby represses gene expression (424). However, whether this effect is directly attributable to transcriptional control via REST has not been addressed. Altered occupancy of deregulated genes by REST in HD mouse models nonetheless makes it an interesting target for future investigations (427)

Other histone PTMs have also recently been implicated in HD. In a cell culture model of HD, the interaction between Htt and hPRC1L E3 ubiquitin ligase complex was shown to be disrupted and lead to an accumulation of monoubiquityl histone H2A (uH2A) (428). Consistently, uH2A level is increased at the promoter of repressed genes in the brain of transgenic R6/2 HD mouse, while uH2B is decreased at these genes. This is linked to H3K9 methylation-dependent gene repression by uH2A, and H3K4 methylation and gene expression by uH2B (428). These findings suggest a functional balance between uH2A and uH2B that is disturbed in HD. Thus, altered histone acetylation, methylation and ubiquitylation seem to act in concert in HD. Despite some divergence in their alteration in different models, the observed epigenetic dysfunctions reduce gene expression. Treatment with HDAC inhibitors have yielded promising initial results in mouse models of HD, providing neuronal protection by correcting general mRNA abnormalities through modification of histones (395, 419). However, application of HDAC inhibitors will need to be refined and made more specific in the future, and other drugs targeting histone methylation and/or ubiquitylation may also be envisaged.

## 9.5. Concluding remarks

Epigenetic mechanisms are critical regulators of the development and proper functioning of the nervous system. DNA methylation and histone PTMs in particular,

play prominent roles in the control of gene activity and expression across multiple stages of development, in adulthood and during aging. Their involvement in the dysfunctions of neuronal circuits underlying complex neurodegenerative processes and diseases offers novel perspectives for a better understanding of the causes and mechanisms of these diseases. But, the nature of epigenetic states and their precise involvement remain to be elucidated (429, 430). This shall be aided by technological development and the establishment of novel methods for detecting and quantifying epigenetic marks in the nervous system and blood (more accessible tissue) (431, 432). Ultimately, a better profiling of epigenetic alterations in patients may provide new options for potential diagnostic and for the development of epigenetic drug treatment (433). In this respect, the use of epigenetic drugs will require major progress in their selectivity and safety, and holds great prospects for addressing some of the key questions on the mechanisms of brain functions and their alterations.

## **9.6. Acknowledgements**

We thank Dr Hans Welzl for critical reading of the manuscript. The lab of I.M. Mansuy is supported by the University Zürich, the Swiss Federal Institute of Technology Zürich, the Swiss National Science Foundation, Roche, the National Center of Competence in Research Neural Plasticity and Repair. K. Gapp is supported by a DOC-fFORTE Fellowship from the Austrian Academy of Science.

## **9.7. Authors` contribution**

K.G. and J.B. wrote the section on imprinting disorders. B.W. wrote the section on Alzheimer's diseases and Aging and age-related cognitive decline. K.G. wrote all remaining sections. I.M. supervised writing.

## **10. Appendix 3: Acknowledgements**

Dear Isabelle, I would like to thank you for this experience that has allowed me to grow very much. In every respect, these last 4 years have been very challenging, but you gave me the freedom to try out whatever I wanted, where not scared to let me work on very risky projects and importantly did not loose the patience when correcting my limited writing skills.

Juanito, I think without you I would not have made it. You were not only my scientific, but also my psychological mentor, desk neighbor, tranquilizer, patriotic buddy, friend. You taught me much more than pure science. If one person in my opinion deserves to be called an all around mentor that's you.

Minoo and Carmen for their scientific support and their kind and encouraging words, after every committee meeting and in other occasions, that helped to keep me motivated when already very frustrated. Pawel and David Wolfer Eric Miska for our great collaborations. Alon Chen, Melly Oitzl for their thoughts on how to improve my projects and interpret my data.

Maria, there is little that I would not want to thank Maria for. Not only did you help me out scientifically, but you were also one of the biggest mental supports for any matter.

Franci, the bright sardinian sun on the dark lab horizon! I was so happy to have you around, especially in the early days. I've always considered you as a big support, professionally as well as mentally. Andrea, the time at HIFO without you wasn't the same anymore. I am missing our tours to Kaufleuten for book readings and concerts so much! Thank you for giving me insight into the to me otherwise obscure world of proteomics and all our discussions on how to pimp sperm. I am sure the time will come for our company... Sofia thank you for being my first friend in the lab. Elo, Lukas, Ali, I never imagined that the working environment could be so much fun. You really changed my view of the lab. I know now that in the next lab I will try to hide my personality, not to be target to any personal lunch discussion. Ali you still owe me something. Melissa thanks for all our chats during smoking and the insight you gave me on how to understand Isabelle. Lubka thank you for being the sunshine in the lab, always having a hand for everyone and spoiling us with your delicious lemon cakes.

Sarah what would the last 4 years have been without our psychological analysis and your support during our lunches and travel, not to mention your kind help with Isabelle's favorite model system the cells. Flora thanks for our numerous attempts to go for sports and travel together to Lausanne. Ajmal and Naddl for digging in the powder. Tamara thanks for your introduction to the MSUS model. Saray, I am so

proud of you. Thanks for being my best and only Diploma student. Thanks to you and Joana for the good time in Sardinia and for the neighborhood support! Gregi, you were a superbe stOdent eeeeehhh. Remember me when you get the Nobelprize for epigenetic evolution, or will you finally end up as a teacher – as you always dreamt off.

Special thanks also to my friends in Zürich, my yoga girls Moni and Michi(s), to Fernando especially for introducing me to Moni. Guimo, Christoph and Audrey, Patrick and Isa, Irene, David and also to those far in distance, but close in mind Hubi, Dani, Caro, Manu and Hannah. You all constantly reminded me that work is not the most important thing in life and pulled me back to balance when I was down. Also many thanks to Heiko for being the best animal care taker ever and to Hans Welz for his scientific and moral support. I further want to thank some people without whom I would not have even been able to start the phd here: Peter Berger, Ortrun Mittelsten Scheid, Isabel Liste and Harald Mori.

I owe the most gratitude to my close family Jasmin and Mama, but also to Isa, Janko, and Peter. Last but not least, Alberto you know that without you I would have stopped my phd probably very early on. Thank you for teaching me stats, showing me what matters in life, keeping me on the ground, preventing me from a burn out and simply for being there for me.

## Appendix 4: Curriculum Vitae

Gapp Katharina

*Birthdate:* 14 06 1984

*Nationalities:* austrian, swiss

*e-mail:* gapp@hifo.uzh.ch

*tel:* 0041774359186

*address:* Affolternstrasse 146, 8050 Zürich



### WORKING EXPERIENCES

02/2010 - 12/2013	Phd employment in the molecular cognition laboratory of Prof. Isabelle Mansuy in the Neuroscience Center Zürich University of Zürich
10/2008-09/2009	Diploma thesis under the supervision of Prof. Isabel Liste in the Neurobiology section of the Centro de Biología Molecular Severo Ochoa (Cantoblanco, Madrid)
03/2008-05/2008	Working Experience In the laboratory of Alberto Martínez-Serrano, under supervision of Dr.Claudia Castillo in the Neurobiology section of the Centro de Biología Molecular Severo Ochoa (Cantoblanco, Madrid)
08/2007-09/2007	Working experience in the laboratory of Prof. Peter Berger in the Endocrinology section of the Austrian academy of science, Institute for Ageing research
07/2005-09/2005	Working experience in the Prinz-Eugen-Pharmacy, Innsbruck (Austria)
07/2004-08/2004	Working experience in the pharma wholesale company and pharmacy Koegl, Innsbruck (Austria)
07/2002-06/2003	Social voluntary work in Nicaragua : Parque Marítimo Playa el Coco (teaching english and computer applications)

### STUDIES

02/2010-12/2013	Phd program for Neurosciences at ETH Zürich (Switzerland)
02/2008-06/2008	Universidad Autónoma de Madrid (Spain) Erasmus exchange semester
2003 - 2009	University of Vienna (Austria) : studies of Molecular Biology with majors in Genetics, Molecular Medicine and Neurobiology
1994 - 2002	Bundesrealgymnasium Adolf-Pichler-Platz, Innsbruck (Austria), Science- orientated «Matura »; Fachbereichsarbeit [specialized paper] in Biology (title : « Arterhaltung gegen Fehleranhaeufung » [species preservation against accumulation of errors] )

## **AWARDS/ SCHOLARSHIPS**

11/2013	Scholarship for attendance of the Society of Neuroscience meeting by Swiss Society of Neuroscience & Hartmann Müller-Stiftung for medical research
04/2013	Scholarship for attendance of the Symposium Non-coding RNA, epigenetic and transgenerational inheritance Cambridge UK by Neuroscience Center Zürich
04/2013	Scholarship for attendance of the Symposium Programming Obesity Cambridge UK by Abcam
09/2012	Scholarship for Frontiers in Stress and Cognition: From Molecules to Behavior by Swiss Laboratory Animal Science Association
04/2012	Scholarship for the attendance of the EMBO practical course on analysis of small non coding RNAs by Neuroscience center Zürich
11/2011	Scholarship for attendance of the Society of Neuroscience annual meeting Washington by Neuroscience Center Zürich
02/2011	fFORTE grant for female doctoral students of natural sciences of the Austrian academy of science
10/2010	Scholarship for the VIBes 2010 symposium in biosciences by VIB
2009	Diploma University of Vienna with distinction
2007	Leonardo da Vinci scholarship for internship in the laboratory of Alberto Martinez SerranoUniversidad Autonoma de Madrid by OeAD (Austrian International Exchange Services)
2008	Erasmus scholarship for exchange semester at the Universidad Autonoma de Madrid by OeAD (Austrian International Exchange Services)

## **PUBLICATIONS**

- Online article: Gapp, K.; (2014) Sperma reloaded: Gene, Umwelt, Vererbung.; <http://science.orf.at/stories/1730226/>
- Book chapter: Woldemichael B., Bohacek J., Gapp K. and Mansuy IM.; (2013) Epigenetics of Learning and Plasticity; Progress in Molecular Biology and Translational Science in press
- Review: Gapp, K., Woldemichael B., Mansuy IM.; (2012) Epigenetic regulation in Neurodevelopment and Neurodegenerative disease; Neuroscience Neuroscience. 2012 Dec 19. pii: S0306-4522(12)01151-7. doi: 10.1016/j.neuroscience.2012.11.040.
- Review: Bohacek J., Gapp, K., Saab B., Mansuy IM.; (2012) Transgenerational Epigenetic Effects on Brain Functions; Biological Psychiatry Biol Psychiatry. 2012 Oct 9. pii: S0006-3223(12)00729-9. doi: 10.1016/j.biopsych.2012.08.019.
- Article: Johannes Bohacek, Katharina Gapp, Isabelle M. Mansuy; (2011) Gestresste Eltern – Gestresste Enkeln. Laborwelt. Nr. 3/2011-12.Jahrgang: 6-9

## **ORAL PRESENTATIONS**

11/2013	Society for Neuroscience annual meeting; title: Implication of sperm RNAs in the inheritance of the effects of early traumatic stress in mice; San Diego, CA, USA
11/2012	ETH day; title: Wie traumatische Kindheitserlebnisse erbliche Hirnkrankheiten auslösen können; Zürich, Switzerland
01/2011	Invited Presenation at Institute of Biology Valrose; Universite Nice Sophia Antipolis: Title: MSUS treatment and impulsivity

## **SELECTED POSTER PRESENTATIONS**

- Poster: Gapp K., Bohacek J., Pelczar P., Brunner A., Prados J., Farinelli L. and Mansuy IM.; Implication of sperm miRNAs in the transgenerational effects of early traumatic stress in mammals; Symposium Non-coding RNA, epigenetic and transgeneartional inheritance; Abcam symposium; Cambridge; UK; 2013
- Poster: Gapp K., Bohacek J., and Mansuy IM.: Environmental enrichment counteracts the transgenerational transmission of the effects of early life stress on behavior; Swiss Society of Neuroscience Meeting 2013, Geneva, Switzerland
- Poster: Gapp, Soldado Magraner S., Mohanna s., Bohacek J., Mansuy IM: Early traumatic stress modulates behavioral control in mice across generations; Neuroscience Center Zürich symposium, Zürich Switzerland; 2012
- Poster: Gapp, Soldado Magraner S., Mohanna s., Bohacek J., Mansuy IM: Early traumatic stress modulates behavioral control in mice across generations; EBBS Frontiers in stress and cognition, Ascona, Switzerland 2012
- Poster: Gapp K., Soldado Magraner S., Mohanna s., Bohacek J., Mansuy IM: Early chronic stress alters behavioral control in mice; Swiss Society of Neuroscience Meeting 2012, Zürich, Switzerland
- Poster: Gapp K., Soldado Magraner S., Corcoba A., Mohanna s., Bohacek J., Mansuy IM: Analysis on the impact of early chronic stress on behavioral control; Society for Neuroscience Meeting 2011, Washington DC, USA
- Poster: Gapp K., Soldado Magraner S., Corcoba A., Mohanna s., Bohacek J., Mansuy IM: Analysis on the impact of early chronic stress on behavioral control; Neuroscience Center Zürich symposium 2011 2011, Zürich, Switzerland
- Poster: Bohacek J., Coiret G., Mirante O., Iniguez AL., Steiner G., Kashuk C., Manuella F., Gapp K., Moreau JL., Mansuy IM.; Genome-wide Analyses of the Transgenerational Impact of Early Life Stress in Mice; Swiss Society of Neuroscience Meeting 2011, Basel, Switzerland
- Poster: Gapp K., Isabelle Mansuy; Study of the Impact of Early Chronic Stress on Behavior Across Generations in Mice); Award of fFORTE at the Austrian Academy of Science 2011, Vienna, Austria
- Poster: Gapp K., Satrustegui J., Martínez-Serrano A., Liste I.; Role of Aralar (a Calcium-binding mitochondrial aspartate-glutamate carrier) in pluripotency and dopaminergic neuronal differentiation; P091, V Congreso de la sociedad española de terapia genica y celular, 2009, Granada, Spain
- Poster: Castillo C.G., Bueno C., Moreno B., Gapp K., Courtois E. T. C., Ramos M., Martínez-Serrano; Characterisation of human neural stem cell lines transfected with homologous recombination vectors: in vitro and in vivo studies; 622.26/B26, Society of neuroscience congress, 2008, Washington, USA

## **TEACHING EXPERIENCES/ PUBLIC RELATIONS**

06/2013	Vaccariat at highschooll Unterstrass as a biology teacher
04/2013-06/2013	Supervision of a Master's project carried out by Gregoire Vernaz
11/2012	Modul Epigenetik: Der Mensch zwischen Determinismus und Umwelt, Heilpädagogische Hochschule Zürich
05/2012-08-2012	Supervision of a internship student, Megan Frugoli
09/2010-06-2011	Supervision of a Master thesis carried out by Saray Soldado Magraner
03/2010/2011/2012	Supervision of practical ETHZ Block Course/Practical 376-1346-00 G Neurobiology
08/2011	Scientifica 2011 Zürcher Wissenschaftstage (public education activity)
07/2002-06-2003	Social voluntary work in Nicaragua : Parque Maritimo Playa el Coco (teaching english and computer applications, accounting)

## **LANGUAGES**

- German (mother tongue)
- English (written and spoken high level)
- Spanish (written and spoken high level)
- French (written and spoken intermediate level)

## **SKILLS**

Microsoft Office, Adobe Photoshop, Adobe Indesign, Adobe Illustrator, Adobe Premiere Pro, Mac, SPSS, Prism, accounting, scientific writing, funds acquisition