DOCUMENT SUMMARY This 2010 research paper by Franklin et al. investigates the transgenerational effects of early life stress in mice. The study demonstrates that chronic, unpredictable maternal separation in male mice leads to depressive-like behaviors and altered stress responses, which are then transmitted to their offspring across multiple generations, even when the offspring are raised normally. The mechanism for this transmission is shown to be epigenetic, involving specific changes in **DNA methylation** in the male **germline** that are also found in the brains of their descendants.

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FORMATTED CONTENT

Epigenetic Transmission of the Impact of Early Stress Across Generations

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Abstract

Background: Traumatic experiences in early life are risk factors for the development of behavioral and emotional disorders. Such disorders can persist through adulthood and have often been reported to be transmitted across generations.

Methods: To investigate the transgenerational effect of early stress, mice were exposed to chronic and unpredictable **maternal separation** from postnatal day 1 to 14.

Results: We show that chronic and unpredictable **maternal separation** induces **depressive-like behaviors** and alters the behavioral response to aversive environments in the separated animals when adult. Most of the behavioral alterations are further expressed by the offspring of males subjected to **maternal separation**, despite the fact that these males are reared normally. Chronic and unpredictable **maternal separation** also alters the profile of **DNA methylation** in the promoter of several candidate genes in the **germline** of the separated males. Comparable changes in **DNA methylation** are also present in the brain of the offspring and are associated with altered gene expression.

Conclusions: These findings highlight the negative impact of early stress on behavioral responses across generations and on the regulation of **DNA methylation** in the **germline**.

Contemporary models of developmental psychopathology suggest that adverse environmental, psychosocial, or physical experiences during early life are predisposing factors for the development of behavioral and emotional disorders in adulthood. In humans, primates, and rodents, insecure attachment and unreliable, disorganized, poor maternal care negatively influence appropriate behavioral responses and cause maladaptive behaviors (1-3). Epidemiological studies have further shown that the offspring of people with such behavioral alterations, and sometimes the generation following that offspring, are often similarly affected even if they themselves, did not experience the trauma (4-7).

> The observation that stress-induced behavioral alterations can be transmitted across generations is intriguing and of fundamental importance, yet this phenomenon has not been well studied in mammals.

Because it implicates environmental factors, it is suggested to be of **epigenetic** nature (8,9).

Here, using an experimental paradigm for chronic and unpredictable stress in early life in C57BL/6 mice, we provide evidence that the transgenerational transmission of complex behavioral alterations induced by early stress can be modeled in animals. We show that chronic and unpredictable **maternal separation** during early postnatal development in mice induces **depressive-like behaviors** and alters the animals' response to novel and aversive environments. Most of the observed behavioral alterations are transmitted to the offspring of males subjected to **maternal separation** and to the subsequent generation. Further to perturbing behavior, early stress is also shown to alter **DNA methylation** of several candidate genes in the **germline** of males subjected to **maternal separation**, as well as in the brain and, for some genes, the **germline** of the offspring. These results suggest that early stress persistently alters behavior and modifies the **epigenetic** profile of genes across generations, providing a behavioral and molecular correlate to complex traits induced by early stress.

Methods and Materials

Animals

C57B16/J females and males (2.5 months) were obtained from Elevage Janvier (Le Genest Saint Isle, France) and maintained in a temperature- and humidity-controlled facility on a 12 hour reversed light-dark cycle with food and water ad libitum. All procedures were carried out in accordance with Swiss cantonal regulations for animal experimentation.

Maternal Separation

Dams and litters were subjected to unpredictable **maternal separation** combined with unpredictable maternal stress (**MSUS**) for 3 hours daily from postnatal day 1 through 14 (PND 1-14) or were left undisturbed except for a cage change once a week (control) until weaning (PND21). Maternal behaviors were monitored during the first 2 weeks after delivery by noting the behavior occurring each minute during 30 min, three times per day (shortly before, shortly after, and 2-3 hours after separation; see Supplement 1. Supplementary Methods). Once

weaned, pups were reared in social groups (3-4 mice/cage) composed of animals subjected to a similar treatment but from different dams to avoid litter effects. To produce a second generation, first-generation (F1) control and **MSUS** males were mated with naive primiparous C57B16/J females following behavioral testing. Mating occurred over 1 week, after which the males were removed from the breeding cage such that they never had any contact with the offspring. Maternal behaviors were scored during the first postnatal week. To produce a third generation, second-generation (F2) control and **MSUS** males were mated with naive primiparous C57B16/J females following behavioral testing, similar to F1. The F2 and F3 offspring were weaned at PND21 and reared in mixed social groups, similar to F1. Unpaired t test followed by Fisher's Protected Least Significant Difference post hoc tests when appropriate were used to analyze maternal care data in FO dams and dams mated to F1 sires.

Behavioral Testing

In all tests, the experimenter was blind to treatment, and behaviors were monitored by direct observation and videotrack-ing (Ethovision, Noldus Information Technology Wageningen, The Netherlands). Behaviors were assessed in adult F1, F2, or F3 animals (3-8 months old). Each animal was tested on a maximum of five tasks, 1 to 2 weeks apart, starting with the least aversive task, under indirect, dim red light. The **forced swim test** and **sucrose consumption** were used to assess **depressive-like behaviors**, and the **free exploratory paradigm** and **open field** were used to assess behavioral response to novel or aversive environments. The antidepressant desipramine was used to reverse **depressive-like behaviors**. For details, see Supplement 1, Supplementary Methods.

DNA Methylation Assays

Pyrosequencing (Qiagen, Hilden, Germany) was used to quantify methylation of CpG islands (defined as ≥60% CG dinucleotides, an observed vs. expected CpG dinucleotide ratio of≥ 60%, and sequence window ≥ 150 bp) (10-12) in candidate genes in sperm from F1 males.

Sample Preparation and Bisulfite Treatment Genomic DNA was prepared from sperm collected from the caudal epididymis of F1 and F2 males, similar to Rakyan et al. (13), and cortex (bregma -2.3) collected from F2 female offspring (DNeasy Blood and Tissue Kit, Qiagen). Purified DNA was processed by bisulfite modification (EZ **DNA Methylation**-Gold Kit, Zymo Research, Irvine, California).

Pyrosequencing The percentage of methylated alleles at each CG site was quantified in bisulfite-converted DNA by pyrosequencing, a high-resolution method for quantitative analyses of the relative proportion of methylated versus unmethylated nucleotides (see Supplement 1, Supplementary Methods). Interassay variability of methylation values was less than 5%, consistent with that previously reported (14).

In Vitro Methylation Assays Acute mouse brain slices were treated with the DNA methyltransferase (**DNMT**) inhibitor zebularine, a hypomethylating agent, and methylation levels were quantified with methylation-specific primers, as described in Levenson et al. (15) (see Supplement, 1 Supplementary Methods).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

DNasel-treated RNA isolated from cortex (RNeasy Mini Kit; Qiagen) was used for reverse transcription (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; see Supplement 1, Supplementary Methods).

Results

To evaluate the extent to which early chronic stress constitutes a risk factor for persistent behavioral alterations, we established a model of unpredictable postnatal stress in mice (Figure 1A). Primiparous C57BL/6 females (FO) and males were bred, and their litters (F1) were subjected to unpredictable **maternal separation** combined with unpredictable maternal stress (**MSUS**) for 3 hours daily from PND 1 to 14. We assessed the impact of the manipulation on the subsequent offspring by breeding adult F1 **MSUS** and control males with wild-type females and produced second-generation (F2) mice under normal rearing conditions (no **maternal separation** or stress involved). F2 **MSUS** and control. males were further bred with wild-type females to produce third-generation (F3) animals, again under normal rearing conditions. Maternal behaviors were monitored daily in FO females and naive females bred to F1 males to 1) evaluate the immediate effect of **MSUS** on maternal care provided by FO dams to F1 pups and 2) control for any potential environmental influence on transmission to F2 pups. Among the multiple parameters examined, arched-back nursing (ABN) and ABN associated with licking-grooming were used as an index of active maternal care, and time off nest as an index of absence of care (16,17). The overall resulting deficit in maternal care induced by **MSUS** was apparent in FO dams primarily during the first postnatal week, when the pups are the most dependent on maternal attention (Figure 1B and 1C). However, despite deficient maternal care, the separated F1 pups grew normally and had normal weight at weaning (PND21) (Supplement 1, Figure S1). As expected, naive dams bred to F1 **MSUS** males provided normal maternal care to F2 offspring (Figure ID and 1E). Consistently, F2 **MSUS** offspring grew normally and had body weight comparable to control animals at weaning (Supplement 1, Figure S1).

Because early stress has been reported to play a role in the development of depressive symptoms in animals and humans, we investigated whether **MSUS** animals showed **depressive-like behaviors** (18-20). We used classical paradigms that allow the assessment of behavioral despair and learned helplessness in rodents. In a **forced swim test** (21), we observed that F1 **MSUS** males spent significantly more time floating (Figure 2A), suggesting depressive-like behavior. F1 **MSUS** females spent significantly less time floating than control females as opposed to males (data not shown), confirming previous findings that **maternal separation** has a negative influence primarily in males (22). We confirmed the depressive-like phenotype in F1 **MSUS** males using a tail suspension test and observed that the animals also had increased time spent immobile on this task (data not shown). We next examined whether this behavioral phenotype was transmitted to the following generation and tested F2 offspring of F1 **MSUS** males in the same experimental conditions. Female F2 offspring had a comparable increase in floating time on the **forced swim test** (Figure 2B), suggesting transmission of this trait.

*Figure 1 Caption: Experimental design and maternal care provided to first- and second-generation (F1 and F2) **maternal separation** with unpredictable maternal stress (**MSUS**) and control pups. (A) Experimental design used to study the impact of **MSUS** on behavior and its transmission across generations. (B) Dams undergoing **MSUS** spent less time actively nursing and (C) more time off nest than control dams during the first week postdelivery. (D, E) Similar*

*level of maternal care was provided to F2 **MSUS** and control mice derived from F1 **MSUS** males.*

*Figure 2 Caption: **Depressive-like behaviors** in first-generation (F1) males and second-and third-generation (F2 and F3) offspring. (A) In the **forced swim test**, there was increased time spent floating in F1 **MSUS** vs. control males. (B) Increased floating in F2 **MSUS** females but not F2 **MSUS** males. (C) Increased floating in F3 **MSUS** males but not females. (D) Lower sucrose intake in F1 **MSUS** vs. F1 control males. (E, F) No significant change in sucrose consumption in F2 or F3 animals.*

On both the **forced swim** and tail suspension test, the increased time spent floating or immobile could be reversed by treatment with the antidepressant desipramine in F2 **MSUS** mice, confirming that it reflected a depressive-like behavior (Figure 3). This behavior was not observed in F2 males, however, suggesting differential and sex-dependent expression of this trait, akin to that reported in human (23-27). Although F2 males did not express obvious depressive symptoms, we nonetheless examined whether their progeny would display these symptoms. For this, F3 offspring obtained from F2 males were tested on the **forced swim** task. Strikingly, F3 male offspring expressed similar depressive symptoms as F1 males and spent more time floating in the test (Figure 2C).

> These results therefore suggest that **depressive-like symptoms** can be transmitted across several generations but with a complex and sex-specific mode of transmission.

Such complex transmission is currently not understood but is reminiscent of that observed in humans (6,7).

Anhedonia—the inability to enjoy pleasurable stimuli—is another symptom commonly associated with depression in humans (26). We assessed whether this trait was present in our mouse model using a **sucrose consumption** test. On this test, a decrease in sucrose intake reflects anhedonia (27). F1 **MSUS** males (Figure 2D), but not females (data not shown), were found to consume less sucrose than control mice, suggesting anhedonia. However, unlike immobility on the **forced swim** and tail suspension tests, this trait was not transmitted to the offspring as sucrose consumption was not significantly altered in F2 **MSUS** females or males (only slightly decreased) or in F3 **MSUS** animals (Figure 2E and 2F). These results suggest that anhedonia can be altered by direct early stress but has reduced penetrance across generations.

Stress sensitivity is another key factor for predisposition to depression and is known to be affected by early trauma (28,29). To assess whether stress sensitivity was altered in our **MSUS** model, we tested the animals on behavioral paradigms exposing them to unfamiliar or aversive conditions. We first used the **free exploratory paradigm**, a mildly stressful test that challenges behavioral response upon exposure to an unfamiliar environment (30). On this task, F1 **MSUS** males had shorter latency to enter the unfamiliar areas of the arena than control animals (Figure 4A). This effect was not due to increased locomotor activity or higher arousal because F1 **MSUS** animals had comparable number of entries in the familiar areas and covered a total distance similar to F1 controls during testing (Supplement 1, Figure S2A and S2B). When placed in an **open field**, a slightly more stressful test, F1 **MSUS** males consistently showed a propensity to enter the aversive center of the field sooner than control mice (Figure 4D). Again, this was not due to an alteration in locomotor activity because the animals covered a total distance similar to control mice (Supplement 1, Figure S2C).

We next examined whether these traits can be transmitted to the offspring of F1 **MSUS** males, and for this we tested F2 and F3 animals. On both the **free exploratory paradigm** and the **open field**, we observed that the female offspring from F1 **MSUS** males had a shorter latency to enter the unfamiliar and aversive areas on both tasks (Figure 4B and 4E). The decrease in latency was comparable to that observed in their F1 fathers. However, the male offspring had latencies comparable to that observed in control animals on both tests. Nonetheless, similar to **depressive-like behaviors**, the reduced latency to enter unfamiliar and aversive areas was also expressed by animals from the F3 offspring on both the **free exploratory paradigm** and the **open field**. The reduction in latency in F3 animals was comparable to that in F1 and F2 animals (Figure 4C and F). These results corroborate the data on the **forced swim test** and further suggest a sex-dependent transmission of the effect of **MSUS** across generations.

*Figure 3 Caption: Reversal of **depressive-like behaviors** in second-generation (F2) **MSUS** mice with acute and chronic antidepressant treatment. In both the **forced swim test** and **tail suspension test**, increased immobility in saline-treated F2 **MSUS** females was reversed by both acute and chronic treatment with desipramine.*

*Figure 4 Caption: Altered behavioral response in first-generation (F1) males and second- and third-generation (F2 and F3) offspring. The study found reduced latency to enter unfamiliar/aversive areas in the **free exploratory paradigm** and **open field** test. This was observed in (A, D) F1 **MSUS** males, (B, E) F2 **MSUS** females (but not males), and (C, F) F3 **MSUS** females (but not males), compared to controls.*

Although an alteration of approach-avoidance behaviors in unfamiliar areas may be interpreted as reduced anxiety, the fact that these behaviors were expressed on initial exposure to these areas and affected the latency to first enter instead suggested a deficit in behavioral control. To investigate this possibility, F2 **MSUS** and control female mice were repeatedly placed in an **open field** for 2 days, which eliminated the notion of novelty across time. Although F2 **MSUS** females initially covered a larger proportion of their total distance in the aversive center of the field, this proportion decreased on repeated exposure to the same **open field** to similar levels as control mice (Supplement 1, Figure S3).

> These results therefore suggest that the altered transmitted trait does not reflect reduced anxiety but rather altered response to novelty suggesting a deficit in behavioral control.

In addition to examining the impact of **MSUS** on behavioral responses, we also investigated whether **MSUS** affected molecular processes in the separated animals. We focused specifically on the male **germline** because transmission of the behavioral alterations occurred through males and was independent of maternal care, and the **germline** is the only cellular link between generations. We hypothesized that **MSUS** may be altering **epigenetic** mechanisms—in particular, **DNA methylation**—in sperm cells. **DNA methylation** is known to be established and dynamically regulated during development (31-34). It can persistently alter chromatin and gene expression in many cells, including brain cells, and can be passed across generations (13,32,35-38).

*Figure 5 Caption: Altered methylation of **MeCP2**, **CB1**, and the **CRFR2** CpG island in the first-generation (F1) **germline** and second-generation (F2) brain and decreased mRNA expression in F2 brain. (B, F, J) The study found reduced mRNA expression for **MeCP2**, **CB1**, and **CRFR2** in the F2 **MSUS** brain. (C, D) Increased methylation of the **MeCP2** CpG island was found in the sperm of F1 **MSUS** males and the brain of F2 **MSUS** females. (G, H) Increased methylation of*

*the **CB1** CpG island was found in the sperm of F1 **MSUS** males and the brain of F2 **MSUS** females. (K, L) Reduced methylation of the **CRFR2** CpG island was found in the sperm of F1 **MSUS** males and the brain of F2 **MSUS** females.*

To determine whether **DNA methylation** was altered by early stress in the male **germline**, we examined its level in the promoter of several candidate genes in sperm from F1 **MSUS** males. Candidate genes known to be involved in the **epigenetic** regulation of gene expression, or that are associated with depression or emotional behavior were chosen. They included the gene coding for methyl CpG-binding protein 2 (**MeCP2**), a transcriptional regulator that binds methylated DNA. Other candidates were the serotonin receptor 1A and monoamine oxidase A (**MAOA**), a receptor known to play a major role in depression, and an enzyme that catalyzes the degradation of serotonin, respectively (41,42). The cannabinoid receptor-1 (**CB1**), associated with emotionality in rodents, and corticotrophin-releasing factor receptor 2 (**CRFR2**), a stress hormone receptor, were also examined (42-45).

To quantify the level of methylation of the promoter-associated CpG island within these candidate genes, genomic DNA was extracted from F1 **MSUS** male germ cells and subjected to bisulfite conversion followed by pyrosequencing analyses. These analyses revealed that methylation of the CpG island surrounding the transcription initiation site of **MeCP2** and **CB1** genes was increased in F1 **MSUS** sperm (Figure 5A, 5C, 5E, 5G). In contrast, for the **CRFR2** gene, methylation in a stretch of the CpG island located 5' of the transcription initiation site was decreased (Figure 5I and K). Methylation was not changed in target regions of the 5-HT1A or **MAOA** gene (Supplement 1, Figure S4). These data indicate that **DNA methylation** is altered in both directions and in a gene-specific manner in the **germline** of males subjected to early stress. Because they are present in the **germline**, the changes in **DNA methylation** could potentially be maintained and transmitted to the following generation. To test this hypothesis, we checked the profile of **DNA methylation** of the candidate genes in the brain of the female F2 progeny. Strikingly, a similar hypermethylation of the same stretch of CpGs was observed in both the **MeCP2** and **CB1** genes (Figure 5D and 5H) and a hypomethylation of **CRFR2** CpG island (Figure 5L). These changes in methylation were functionally relevant because they were associated with a decrease in the level of mRNA expression of these genes (Figure 5B, 5F, 5J). In this respect, because **DNA methylation** is generally viewed as a gene-silencing mechanism, a decrease in **MeCP2** and **CB1** mRNA expression was expected, but a decrease in **CRFR2** was surprising. Recently, however, **DNA methylation** was shown to be also associated with gene activation, thus making it plausible that **CRFR2** hypomethylation leads to decreased gene expression (46). We confirmed this point by carrying out in vitro assays for which **CRFR2** gene was hypomethylated in brain extracts using the DNA methyltransferase (**DNMT**) inhibitor zebularine. Quantification of **CRFR2** expression by RT-PCR showed that hypomethylation of the **CRFR2** promoter was associated with reduced **CRFR2** expression and that this effect was dose-dependent (Supplement 1, Figure S5). These results therefore confirm that hypomethylation of the **CRFR2** promoter leads to reduced **CRFR2** expression.

Finally, we also tested whether **DNA methylation** was altered in the **germline** of F2 males. We observed that methylation of **MeCP2** was increased in a similar stretch of DNA as in F1 sperm, and methylation of the **CRFR2** CpG island was decreased in a similar stretch in sperm of F2 **MSUS** males (Supplement 1, Figure S6A,C). Methylation of the **CB1** CpG island was however not significantly changed (Supplement 1. Figure S6B), suggesting potential correction mechanisms.

Overall, the data suggest that early stress alters **DNA methylation** in the male **germline** and that some of the alterations can be maintained and passed to the offspring.

Discussion

Our results provide evidence that chronic and unpredictable stress during early postnatal life leads to **depressive-like behaviors** and alters the response to novel and aversive environments in adult mice. They show that these traits are in part transmitted to the subsequent generations. Transmission occurs through males and affects the offspring in a sex-dependent manner. The data also show that early stress alters **DNA methylation** in the **germline** of the stressed males, with either increased or decreased methylation depending on the locus. These alterations are maintained in the **germline** of the stressed males and are also observed, in part, in the subsequent generation in both the brain and male **germline**.

Early life stress is known to alter behavioral responses in animals and humans during adulthood and has persistent effects on the hypothalamic-pituitary-adrenal axis (47). In particular, maternal deprivation and poor maternal care have been widely reported to perturb neurodevelopmental processes in the nervous system and are a risk factor for the etiology of mood and anxiety disorders (48-50). The behavioral defects induced by **MSUS** in our model are reminiscent of several neuropsychiatric diseases in human. In psychopathology, a predisposition to depression, deficient behavioral control, an inability to respond appropriately to a potential danger, and impulsivity are important indicators of behavioral and psychiatric diseases such as borderline personality disorder, antisocial and mood disorders, attention-deficit/hyperactivity disorder (especially the hyperactive-impulsive type) (51), and drug addiction. These diseases often affect not only the individuals exposed to stress but run in families across generations. The mouse model presented here recapitulates the multiple effects of early stressful conditions on behavior and represents a unique and novel model of heritable behavioral disorders due to early stress.

Previous primate and rodent studies have reported increased **depressive-like behavior** after **maternal separation**, and some also reported instances of increased anxiety (2,52,53). Here, the observations that **MSUS** induces a combination of depressive behaviors and alterations in aspects of behavioral control are novel and have not been previously reported. They may result from the specificity of our experimental paradigm, which distinguishes itself from previous manipulations in its unpredictability. Unpredictability of **maternal separation** was essential to produce a lasting behavioral effect in the offspring. When **maternal separation** was predictable and applied at the same time daily, it had no effect on the offspring's behavior. This was because mouse dams were able to adapt to the separation and anticipated their absence by providing extra care before and after separation, thereby preventing any detrimental effect of the separation (data not shown). It was only when separation was made unpredictable and was combined with unpredictable maternal stress that it had persistent effects on the offspring and following generations. Here, it is also possible that unpredictability and concurrent maternal stress exacerbated the impact of separation, possibly by differentially activating stress pathways or selectively altering circuits involved in the behavioral response to stress.

These results show that early stress affects behavior not only in the stressed animals but also in their offspring across generations. The data indicate that transmission occurs through a complex and sex-dependent mode.

The fact that certain traits, despite not being clearly expressed by parents, can be transmitted and expressed by the progeny, suggests that mice can act as "silent" or asymptomatic carriers of specific behavioral alterations.

This notion of a silent carrier is reminiscent of that reported in humans (6,7,23-25), but its mechanisms are not well understood. The mechanisms for the sex dependence of the expression of behavioral alterations also are not known but may involve sex steroids, previously suggested to modulate the **epigenetic** machinery in the mouse brain differentially (54,55).

Transmission of abnormal behavioral traits by males exposed to early stress is not due to any obvious changes in maternal care provided by wild-type mothers to the F2 offspring. However, the possibility that there may be subtle changes in care, such as altered circadian rhythm or milk content resulting from the brief exposure of these females to F1 **MSUS** males cannot be excluded.

Examples of environmental manipulations or genetic mutations that induce **epigenetic** reprogramming in the **germline** and transmission of disease states have been previously reported in plants, Drosophila, and mammals (5,35,36,56-59). Our findings newly suggest that stressful environmental factors can alter **DNA methylation** in the **germline** and that the alterations can be, in part, maintained across several generations. In male rodent germ cells, **DNA methylation** is acquired over successive prenatal and postnatal stages and is completed only after birth (58,60). These mechanisms of regulation are complex and fairly well defined. In mammals, **DNA methylation** is known to be sensitive to various environmental factors such as chemicals, nutritional factors, and hormonal manipulations. It can also be altered in aging and has been associated with multiple brain diseases and psychiatric disorders, as well as cancer and immune and metabolic disorders (61-64). The mechanisms leading to altered **DNA methylation** in sperm in our mouse model are not known but likely involve multiple factors because both hyper- and hypomethylation were observed. Changes in **DNMTs**, in noncoding RNAs, and/or chromatin remodeling complexes may be involved (31,33,34).

Several specific genes are affected in their **DNA methylation** profile by **MSUS** in a bidirectional and locus-dependent manner. Our study identified only a few genes, but many more are expected to be altered, and **MSUS** most likely has an impact on the epigenome on a global scale. The few genes identified here probably contribute to only a part of the behavioral phenotype and, in combination with other genes, may underlie the complex behavioral phenotype observed in our model. Such multigenic etiology is consistent with that observed in humans (65). The fact that the expression of the candidate genes is affected only moderately in the brain of F2 animals supports the hypothesis that each gene likely contributes to only a part of the behavioral phenotype. It should be noted that the high ratio of candidate genes found to be altered results from the fact that these genes were carefully selected on the basis of their known involvement in gene regulation or behavior and therefore and may not be representative of the entire genome. Finally, although methylation analyses were restricted to the promoter-associated CpG islands of these genes, methylation in intragenic regions may also be altered.

Overall, these findings are the first to demonstrate that postnatal stress in mice can persistently affect behavior across generations and **DNA methylation** in the **germline**. These findings significantly extend previous data showing that **DNA methylation** in the brain is influenced by poor maternal care (66-68) and illustrate the broad detrimental impact of early stress.