Epigenetic Transmission of the Impact of Early Stress Across Generations

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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.

Key Words: Brain, depression, DNA methylation, early stress, epigenetic, 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.

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Methods and Materials

Animals

C57Bl6/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 C57Bl6/J females following behavioral testing. Mat-

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ing 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 C57Bl6/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 F0 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 videotracking (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

DNaseI-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 (F0) 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 F0 females and naive females bred to F1 males to 1) evaluate the immediate effect of MSUS on maternal care provided by F0 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 F0 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 1D 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.

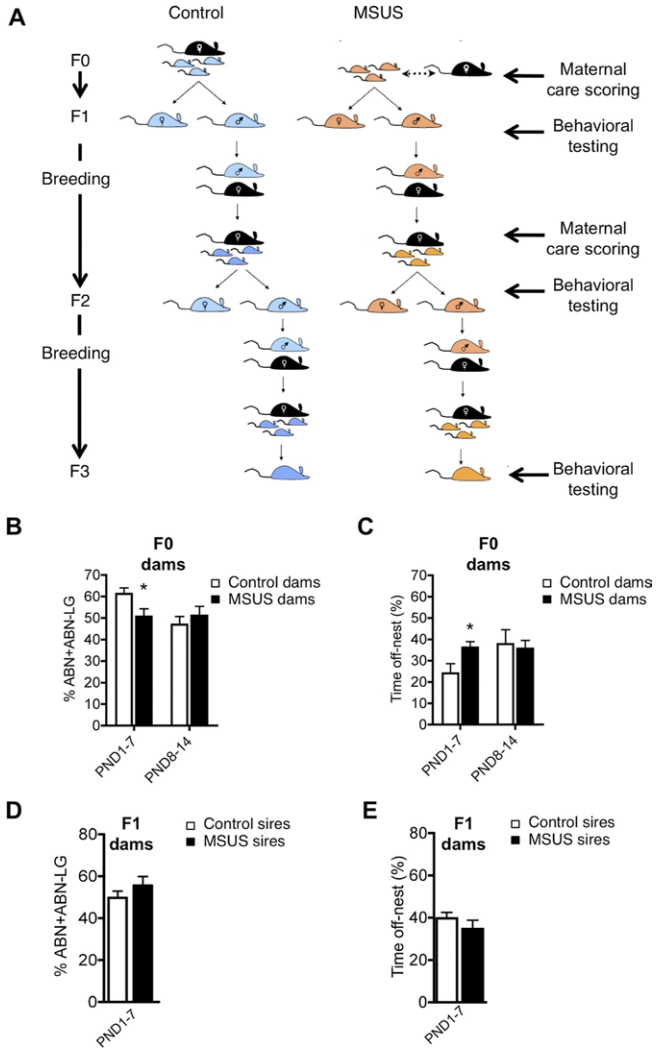

Figure 1. Experimental design and maternal care provided to first- and second-generation (F1 and F2) maternal separation with unpredictable maternal stress (MSUS) and control pups. **(A)** Experimental design used to study the impact of MSUS on behavior and its transmission across generations. C57Bl6/J F0 females (black) bred to C57Bl6/J males were allowed to raise their offspring in normal conditions (control; left, blue) or were subjected to MSUS from postnatal day (PND) 1 to 14 (right, orange), and maternal care was observed. The F1 progeny (females and males) was weaned, raised normally until adulthood, then behaviorally tested. Following testing, adult F1 control (blue, left) and F1 MSUS (orange, right) males were bred to C57Bl6/J females (black), and F2 offspring were raised in normal conditions (no maternal separation or maternal stress). F2 control and MSUS males were bred to C57Bl6/J females, and F3 offspring were raised in normal conditions. The dotted arrow symbolizes separation of dams from pups. Poor maternal care provided to F1 MSUS pups did not result in abnormal weight during postnatal development. Dams undergoing MSUS ($n = 7$) spent **(B)** less time actively nursing [$F(1,13) = 7.25$, $p < .05$] and **(C)** more time off nest [$F(1,13) = 7.69$, $p < .05$] than control dams ($n = 8$) during the first week postdelivery (PND 1–7). Average of maternal care scoring across three 30-min sessions per day. Similar level of maternal care was provided to F2 MSUS and control mice derived from F1 MSUS males. Daily scoring of maternal care from PND 1 to 7 (three 30-min sessions/day) showing similar **(D)** level of active nursing [arched-back nursing (ABN) and ABN associated with licking-grooming (ABN + ABN-LG), in percent; $t(17) = 1.10$, ns] and **(E)** time off-nest [$t(17) = 1.02$, ns; control, $n = 10$; MSUS, $n = 9$]. Maternal care provided to F2 pups was not monitored from PND 8–14 because it had not changed from PND 1–7. *$p < .05$ as indicated by Fisher's Protected Least Significant Difference following two-way analysis of variance.

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

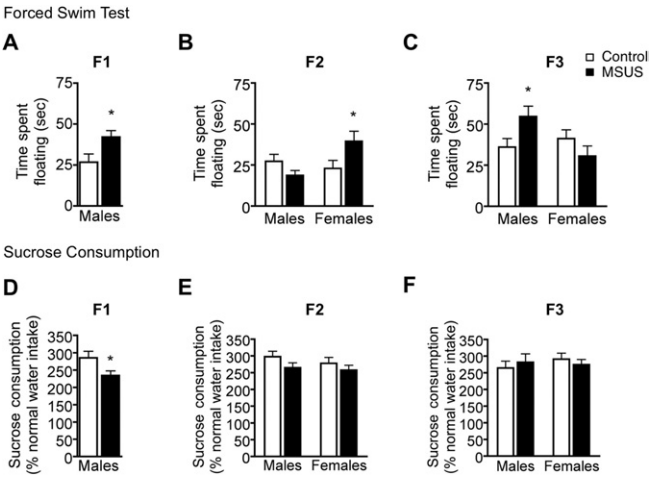

Figure 2. Depressive-like behaviors in first-generation (F1) males and second- and third-generation (F2 and F3) offspring. In the forced swim test, **(A)** there was increased time spent floating in F1 maternal separation with unpredictable maternal stress (MSUS; $n = 29$) vs. control males [control, $n = 14$; $t(42) = 2.25$, $p < .05$]; (**B**) F2 MSUS females ($n = 31$) compared with F2 control females ($n = 28$), but not F2 MSUS males ($n = 29$) compared with F2 control males [$n = 30$; $F(1,114) = 6.95$, $p < .01$]; and **(C)** in F3 MSUS males ($n = 22$) but not females ($n = 18$) compared with controls [males, $n = 20$; females, $n = 19$; $F(1,75) = 6.09$, $p < .05$]. Lower sucrose intake normalized to water intake over 4 days in **(D)** F1 MSUS ($n = 20$) vs. F1 control males [control, $n = 18$; $t(36) = 2.22$, $p < .05$] but not in **(E)** F2 MSUS vs. F2 control mice [F2 control males, $n = 13$; F2 MSUS males, $n = 16$; F2 control females, $n = 16$; F2 MSUS females, $n = 16$; $F(1,57) = 3.07$, $.05 < p < .1$] or **(F)** F3 MSUS and control males or females [F3 control males, $n = 18$; F3 MSUS males, $n = 18$; F3 control females, $n = 19$; F3 MSUS females, $n = 19$; $F(1,70) = .36$, ns]. *$p < .05$ as indicated by unpaired t test or Fisher's Protected Least Significant Difference following two-way analysis of variance.

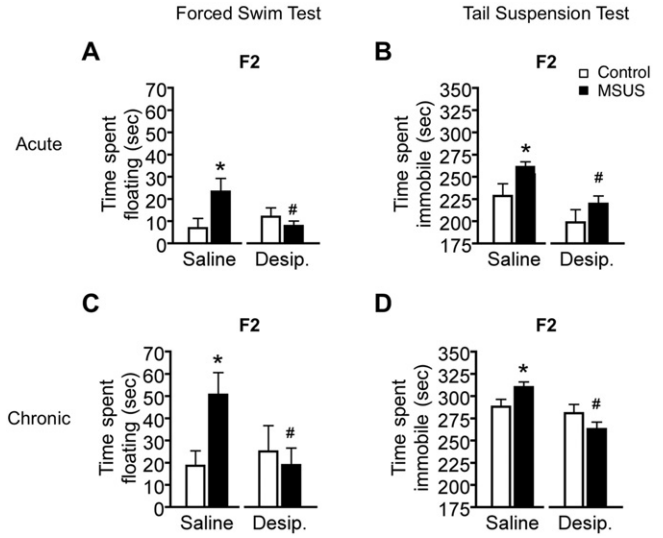

Figure 3. Reversal of depressive-like behaviors in second-generation (F2) maternal separation with unpredictable maternal stress (MSUS) mice with acute and chronic antidepressant treatment. In the forced swim test, increased time spent floating in saline-treated female F2 MSUS ($n = 17$) vs. F2 control ($n = 14$) was reversed by both **(A)** acute (1 day) and **(C)** chronic (14 day) treatment with desipramine [Desip.; F2 MSUS $n = 14$, F2 control, $n = 11$; **(A)** $F(1,52) = 2.93$, $p < .05$; **(C)** $F(1,47) = 3.22$, $p < .05$]. In the tail suspension test, increased time spent immobile in saline-treated female F2 MSUS ($n = 16$) vs. F2 control ($n = 14$) was reversed by both **(B)** acute and **(D)** chronic treatment with desipramine [F2 MSUS, $n = 14$, F2 control, $n = 11$; **(B)** $F(1,50) = 5.93$, $p < .001$; **(D)** $F(1,48) = 7.29$, $p < .001$]. $^{*}p < .05$, MSUS vs. control within generation/drug treatment; $^{\#}p < .05$, saline vs. desipramine within MSUS or control as indicated by Fisher's Protected Least Significant Difference post hoc tests.

(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.

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

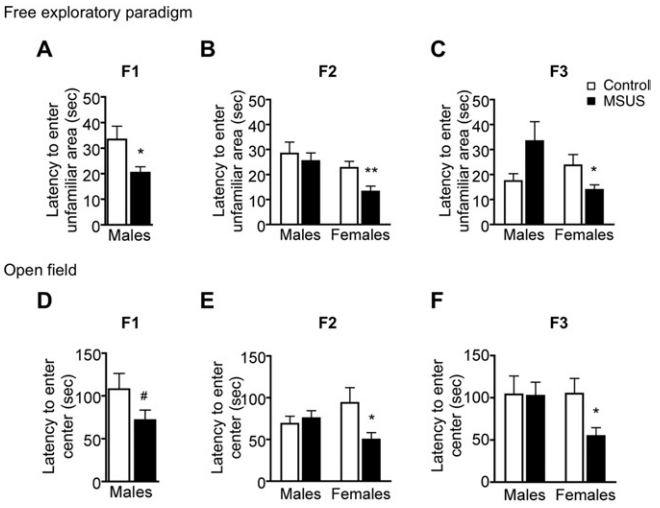

Figure 4. Altered behavioral response in first-generation (F1) males and second- and third-generation (F2 and F3) offspring. Reduced latency to enter unfamiliar areas in **(A)** F1 maternal separation with unpredictable maternal stress (MSUS) males [$n = 41$; $t(64) = 2.60$, $p < .05$], **(B)** F2 MSUS females ($n = 15$) but not F2 MSUS males [$n = 29$; F2, $F(1,84) = 4.74$, $p < .05$], and **(C)** F3 MSUS females ($n = 16$), but not F3 MSUS males ($n = 18$), compared with controls [F1 control males, $n = 25$; F2 control females, $n = 15$; F2 control males, $n = 29$; F3 control females, $n = 18$; F3 control males, $n = 19$; F3, $F(1,67) = 6.94$, $p < .05$] in the free exploratory paradigm. There was a trend for reduced latency to enter the center of the open field in **(D)** F1 MSUS males ($n = 38$; $t(61) = 1.73$, $.05 < p < .1$], significant reduction in **(E)** F2 MSUS females ($n = 15$) but not in F2 MSUS males ($n = 29$; $F(1,83) = 4.88$, $p < .05$], and **(F)** in F3 MSUS females ($n = 17$) but not F3 MSUS males ($n = 19$) compared with controls [F1 controls males, $n = 28$; F2 control females, $n = 15$; F2 control males, $n = 28$; F3 control females, $n = 20$; F3 control males, $n = 18$; F3, $F(1,35) = 5.37$, $p < .05$]. $^{\#}.05 < p < .1$; $^{*}p < .05$; $^{**}p < .01$, as indicated by unpaired *t* test or Fisher's Protected Least Significant Difference following two-way analysis of variance.

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).

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. In addition to its role in Rett syndrome, MeCP2 has also

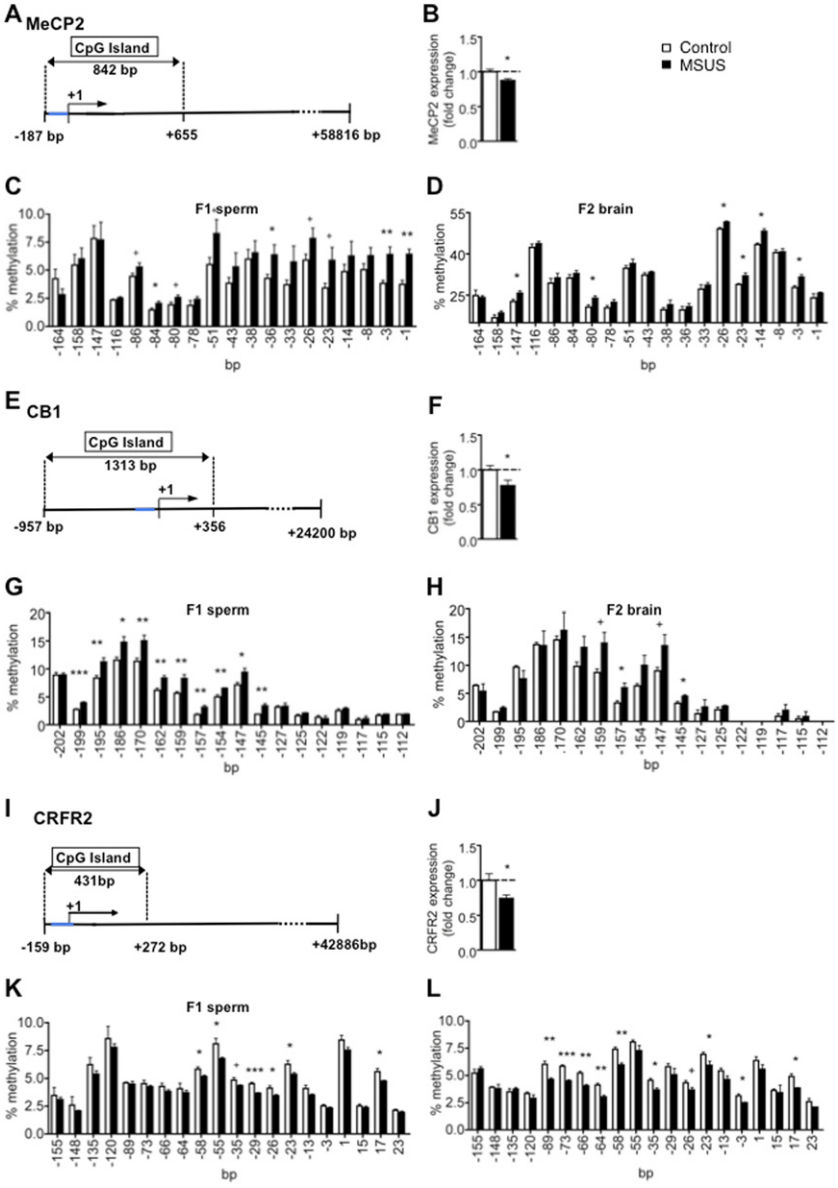

Figure 5. Altered methylation of MeCP2, CB1, and the CRFR2 CpG island in the first-generation (F1) germline and second-generation (F2) brain and decreased mRNA expression in F2 brain. Schematic representation of **(A)** MeCP2, **(E)** CB1, and **(I)** CRFR2 genes showing the CpG island and transcription initiation site. Base-bp annotations are relative to the location of the initiation site. Target sequences used for pyrosequencing to quantify methylation are represented as a blue line. **(B)** Reduced MeCP2 mRNA expression in F2 MSUS ($n = 7$) compared with F2 control ($n = 5$) brain [$t(10) = 2.80$, $p < .05$]. Increased methylation of the MeCP2 CpG island in **(C)** sperm of F1 MSUS males [$F(1,7) = 6.19$, $p < .05$] and **(D)** brain of F2 MSUS females [$F(1,6) = 13.31$, $p = .01$; sperm, $n = 4–5$; brain, $n = 4$]. **(F)** Reduced CB1 mRNA expression in F2 MSUS ($n = 8$) compared with F2 control ($n = 8$) brain [$t(14) = 2.21$, $p < .05$]. Increased methylation of the CB1 CpG island in **(G)** sperm of F1 MSUS males [$F(1,9) = 15.57$, $p < .01$] and **(H)** brain of F2 MSUS females [$F(10,40) = 5.26$, $p < .01$; sperm, $n = 5–6$, brain, $n = 3$]. **(J)** Reduced CRFR2 mRNA expression in F2 MSUS ($n = 11$) compared with F2 control mice [$n = 12$; $t(21) = 2.31$, $p < .05$]. Reduced methylation of the CRFR2 CpG island in **(K)** sperm of F1 MSUS males ($n = 6$) compared with F1 control ($n = 6$) mice [$F(1,10) = 5.31$, $p < .05$] and **(L)** brain in F2 MSUS females [$n = 4$, $F(1,6) = 20.66$, $p < .05$]. $^{+}$ $05 < p < .1$; * $p < .05$; ** $p < .01$; *** $p < .001$, as indicated by Fisher's Protected Least Significant Difference following repeated-measures analysis of variance.

been implicated in the control of stress in mice because its deficiency increases the stress response (39,40). 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-HT_{1A} 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. Males are present for a few days after mating and might affect the very early prenatal development of the progeny. There is, however, no evidence for this hypothesis, and we believe that it is unlikely given the brevity of the exposure and the fact that variable exposure (3–7 days) had a similar impact on behavior and methylation (no increased effect with longer exposure).

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.

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