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Potential of Environmental Enrichment to Prevent Transgenerational Effects of Paternal Trauma

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Adverse experiences in early life are risk factors for the development of behavioral and physiological symptoms that can lead to psychiatric and cognitive disorders later in life. Some of these symptoms can be transmitted to the offspring, in some cases by non-genomic mechanisms involving germ cells. Using a mouse model of unpredictable maternal separation and maternal stress, we show that postnatal trauma alters coping behaviors in adverse conditions in exposed males when adult and in their adult male progeny. The behavioral changes are accompanied by increased glucocorticoid receptor (*GR*) expression and decreased DNA methylation of the *GR* promoter in the hippocampus. DNA methylation is also decreased in sperm cells of exposed males when adult. Transgenerational transmission of behavioral symptoms is prevented by paternal environmental enrichment, an effect associated with the reversal of alterations in *GR* gene expression and DNA methylation in the hippocampus of the male offspring. These findings highlight the influence of both negative and positive environmental factors on behavior across generations and the plasticity of the epigenome across life.
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INTRODUCTION

Exposure to traumatic and stressful events in early life can have physiological and behavioral consequences that persist across the lifespan. Such experiences often increase the risk to develop psychiatric and cognitive disorders in adulthood (Klengel and Binder, 2015). Further, their effects can be transmitted to subsequent generations and affect the offspring similarly to the ancestors in the absence of any trauma or stress exposure (Bohacek and Mansuy, 2015; Metz *et al*, 2015). In some cases, however, negative experiences can have some benefits and lead to better adapted physiological and behavioral responses. Resilience is a form of adaptive response manifested by active or passive coping in adverse conditions (Franklin *et al*, 2012) that can follow previous trauma exposure. For instance, social avoidance is viewed as an active coping behavior that can be manifested by an adult individual after being exposed to traumatic stress in early postnatal life (Franklin *et al*, 2011) or chronic social defeat in adulthood (Kovalenko *et al*, 2014). In the case of early traumatic stress, social avoidance has been shown to be accompanied by altered behavioral flexibility, and both are transmitted to the progeny (Franklin *et al*, 2011; Gapp *et al*, 2014b). Further, some maladaptive behaviors resulting from early trauma can be modulated by the environment; in particular, they can be reversed by conditions such as environmental enrichment (EE) (Leshem and Schulkin, 2012; Schloesser *et al*, 2010). This suggests that the effects of early life trauma, whether detrimental or beneficial, and their transmission might be counteracted by environmental factors later in life.

Mechanistically, the consequences of early exposure to trauma are complex, and implicate a combination of signaling pathways that engage the hypothalamic–pituitary–adrenal (HPA) axis. In this axis, the glucocorticoid receptor (GR) is a major component of stress responses needed for the rapid shut down of the stress response (de Kloet *et al*, 2005a) and for long-term adaptive processes after chronic stress (Jankord and Herman, 2008; Liu *et al*, 1997; Plotsky and Meaney, 1993), especially in the context of active avoidance behavior (Patacchioli *et al*, 1990). *GR* gene is subjected to epigenetic regulation (Weaver *et al*, 2004), and is particularly responsive to environmental factors in early life (Mueller and Bale, 2008).

Here, we used a mouse model of unpredictable maternal separation combined with unpredictable maternal stress (MSUS) to examine the consequences of traumatic stress on

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coping behaviors in adulthood and across generations, and the potential contribution of GR. We show that MSUS affects avoidance behaviors and learning in aversive environments in exposed fathers and their male offspring. This is associated with an increase in *GR* expression in the hippocampus, and with decreased DNA methylation of GR promoter in the hippocampus and in germ cells. We show that transmission of the effects of paternal trauma can be prevented by paternal EE, suggesting a reversibility of these effects.

MATERIALS AND METHODS

Animals

C57Bl/6J male and female mice were maintained under a reverse light–dark cycle in a temperature- and humidity-controlled facility. Three to five mice per cage were housed with water and food *ad libitum* and cages were cleaned once a week. All experimental manipulations were performed during the animals' active cycle and in accordance with guidelines and regulations of the cantonal veterinary office Zurich under license 55/2012.

MSUS Treatment and Breeding

Unpredictable MSUS was conducted as previously described (Franklin *et al*, 2010). Briefly, adult males and females (F0) were mated, males were removed after a few days and females were maintained alone during gestation until delivery. Newborn pups (F1) were separated from their mother unpredictably (any time during the dark cycle) daily for 3 h. During separation, dams were exposed to an additional stress consisting of either 20-min restraint in a Plexiglas tube or 5-min forced swim in cold water (18 °C) applied randomly any time during the 3 h of separation. MSUS was conducted from PND1 to PND14. Dams and pups were then left undisturbed from PND15 until weaning at PND21. About 20–30 females were bred for each MSUS experiment and only dams giving birth less than a week apart of each other were used to obtain F1 pups. Litters with less than four pups were excluded. When adult, F1 control and MSUS males were bred with naive C57Bl/6J females to generate an offspring (F2).

Behavioral Testing

The experimenter was blind to treatment for all behavioral testing and tracking was performed manually and automatically (Viewpoint System, France). Testing started with the least aversive task (light–dark box) when animals were at least 3 months old. All behavioral tests were carried out in males to avoid confounds related to the estrus cycle in females.

Light–Dark Box

Mice were placed in the lit compartment of a plastic box ($40 \times 42 \times 26$ cm) separated from a dark compartment by a divider. The lit (aversive) compartment is large (2/3 of the box), has white walls, and is brightly lit by an overhead lightbulb. The neighboring dark (safe) compartment is small (1/3 of the box), has black walls, and is covered by a black lid. An opening in the divider (5×5 cm) allows the animal to escape from the lit compartment to the dark compartment. Each animal was tested for 10 min and the time spent in each compartment and the latency to enter the dark compartment were measured manually. Light dark box data from the standard housed groups were reported before in Gapp *et al* (2014a).

Active Avoidance Task

Four identical operant conditioning chambers ($15.9 \times 16.5 \times 17.5$ cm) with stainless steel grid floors (TSE Systems, Germany) were placed in sound-insulated boxes. Each chamber is 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 a hole. An additional green cue light is located on the wall on top of the nose-poke module. A house light (2.8 W) is placed on the ceiling, and is turned on during each testing session. The nose-poke module is located on the left part of one wall, which also had a photocell sensor. Each mouse was habituated to the chamber one day for 30 min with the nose-poke module closed. After habituation, animals were tested for active avoidance in a 30-min session with 60 escape trials induced by 0.3 mA foot-shocks delivered every 30 s. Foot-shocks were terminated when the animal had a nose-poke, and lasted a maximum of 10 s. For each trial, the green light was turned on during non-shock periods, and the red light was turned on during shock periods. The sensor located in the nose poke hole recorded the timing of each nose poke with 1 s resolution. Latency to escape and number of escapes were measured in blocks of five trials.

Fixed Ratio Paradigm

Water deprived mice were tested 5 days a week on the fixed ration paradigm and provided water access for 1 h per day. Each operant conditioning chamber was equipped as described above, but further contained a liquid dispenser fitted with a photocell sensor, situated in the middle of the wall left to the nose-poke module. Mice were habituated to the chamber for 30 min, during which 10 µl of water (=liquid reinforcer) were delivered to the liquid dipper every 30 s. Mice were trained on a continuous fixed ratio (FR) schedule for which they had to nose-poke for the delivery of a liquid reinforcer during daily 30-min sessions across 12 days. Activation of the nose-poke led to delivery of 10 µl water in the liquid dipper. Learning was determined by the ratio of total number of nose-pokes over total number of collected drink rewards.

Environmental Enrichment

Control and MSUS mice were placed in social groups ($n = 12$) in an enriched cage from weaning till adulthood. An enriched cage is a large box with two levels ($55 \times 36 \times 19$ cm bottom level, $55 \times 36 \times 11$ cm upper level) (Marlau; Viewpoint). The bottom level is split into two compartments, one containing food pellets and the other providing access to water and containing running wheels,

and a covered/protected area. The upper level has a maze ($35 \times 36 \times 11$ cm) whose shape and configuration are changed three times per week with a total of 12 different options. The box is organized such that animals have to go through the maze to reach the food compartment.

Real-Time Quantitative RT-PCR

DNaseI-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 (qRT-PCR) was performed in an ABI 7500 thermal cycler using TaqMan probes (Applied Biosystems, Foster City, California; *Mm00433832_m1*) as described previously(Franklin *et al*, 2010).

Bisulfite Pyrosequencing

Genomic DNA from the hippocampus, prefrontal cortex, and sperm was extracted with the Allprep (Qiagen) and DNeasy blood and tissue (Qiagen) kit, respectively, according to the manufacturer's instructions. Bisulfite pyrosequencing of offspring hippocampus was performed by EpigenDx, USA. Universally methylated and unmethylated DNA samples (Millipore Bioscience Research Regents) were used as controls. Pyrosequencing of hippocampus, prefrontal cortex and sperm was performed as described previously (Bohacek *et al*, 2014). Amplicons containing the *GR exon1-7* promoter region were generated using a standard PCR protocol, an unmodified forward primer (5′-GGTTTTGTAG GTTGGTTGTTATTT-3′), and a biotin-labeled reverse primer (5′-ATTTCTTTAATTTCTCTTCTCCCTAAC-3′). For high-resolution sequencing, the following sequencing primer was used 5′-TTGTAGGTTGGTTGTTATTTTT-3′.

Samples Preparation, iTRAQ Labeling, Mass Spectrometry Analyses and Protein Quantification

Hippocampi dissected from adult mice were homogenized in 200 µl lysis buffer (100 mM triethyl ammonium bicarbonate pH ~ 8, 0.1% SDS, 2 M urea) by up-and-down strokes using a 27 gauge syringe, sonicated for 2 min, and centrifuged at 13 000 *g* for 10 min to remove insoluble material. Proteins (50 µg) were precipitated using six volumes ice-cold acetone, solubilized, reduced, and cysteines were blocked according to the manufacturer's protocol. Proteins were then digested into peptides with trypsin overnight (1 : 10 enzyme : substrate). Peptides were differentially labeled with iTRAQ 8-plex reagents (Applied Biosystems) according to the manufacturer's protocol then combined. Peptide samples were desalted and separated in-solution with an Agilent 3100 OFFGEL fractionator (Agilent Technologies) according to the manufacturer's protocol. Fractions were acidified by adding 50% acetonitrile (ACN), 1% trifluoroacetic acid (1 : 10), and detergents were removed using ZipTip C18 columns (Millipore). Samples were lyophilized then resuspended in 3% ACN, 0.1% formic acid for LTQ-Orbitrap Velos analysis using collision-induced dissociation (CID), and higher energy collisional dissociation fragmentation as described in Uzozie *et al* (2014). In brief, for the eight most intense signals per cycle above a threshold of 1000, both CID and higher-energy collisional dissociation spectra were acquired in a data-dependent manner. Mascot Distiller 2.4.3.3 (Matrix Science, Boston, MA) was used to generate Mascot generic format peak lists. De-isotoping and peak picking were not performed between 112.5 and 121.5m/z (range containing iTRAQ (isobaric tags for relative and absolute quantitation) reporter ions), and the CID and higher-energy collisional dissociation spectra were merged by summing. Peptide and protein identification was performed with ProteinPilot software v4.5 (AB Sciex) and the Paragon algorithm 4.5.0.0 (Shilov *et al*, 2007), by comparison with the mouse Swissprot/TrEMBL database (downloaded in May 2009). OGE fractions obtained by F1 analyses were searched separately from F2. Paragon method parameters were peptide labeled with iTRAQ 8plex, fixed modification of methyl methanethiosulfonate on Cys (+46 Da), digestion with trypsin, instrument Orbi/FT MS (1–3 ppm) LTQ MS/MS and ID focus on biological modification with the thorough search effort. Proteins with at least one peptide above 90% confidence level as determined by Protein Pilot were recorded. For the estimation of protein abundance ratio, intensity of iTRAQ reporter ion areas for each non-shared, non-discordant, quantifiable, and confidently identified MS/MS spectra was exported from ProteinPilot. For each protein, the sum of each reporter channel was calculated across spectra matched to the same protein. Data were normalized for loading error by bias corrections using ProteinPilot. Statistical significance of differences between means for each group (Control and MSUS mice) was determined on the transformed protein sums (arc sin hyperbolic) using a two-tailed *t*-test. Fold changes were calculated using the group means of the protein sums. Proteins were considered regulated if the *P*-value was <0.05 and fold-change between groups >1.3-fold. Mass spectrometry proteomics data are deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository (identifier PXD004073).

Statistical Analyses

Two-way ANOVAs with treatment (Control and MSUS) and housing (standard and EE) as main factors were used to analyze data of light dark test, qRT-PCR, and bisulfite pyrosequencing. Repeated measurements ANOVAs with treatment and housing as main factors, and within factor of testing session (in blocks of 5 sessions), were used to analyze data of the active avoidance task. Repeated measurements ANOVAs with treatment as a main factor and testing session as within factor were used to analyze data of the FR paradigm. Two-tailed Student's *t*-tests were used to analyze qRT-PCR and bisulfite pyrosequencing data when comparing MSUS and control data in standard housing conditions only. All analyzed data matched the requirements for parametric statistical tests. If variance was not homogeneous between groups, adjusted *P*-value, *t*-value, and degree of freedom were reported. A value was considered outlier when deviating >2 SDs from the group mean, and outlier exclusion criterion was pre-established. Significance was set at $P<0.05$ for all tests. Error bars represent standard error of the mean (SEM).

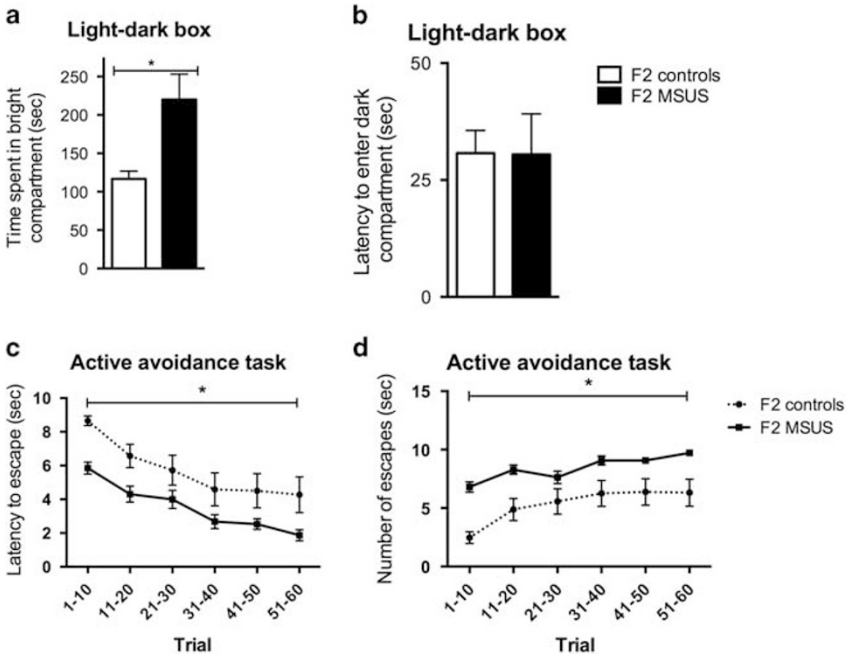

Figure 1 Aversive behavior in the offspring of males exposed to maternal separation combined with unpredictable maternal stress (MSUS) in postnatal life. Performance in a light–dark box shown by (a) time spent in the bright compartment in control and MSUS (Controls $n = 33$, MSUS $n = 36$) and (b) latency to first enter the dark compartment (Controls $n = 34$, MSUS $n = 38$). (c) The latency to escape a foot-shock and (d) the total number of foot-shocks on the active avoidance task (Controls $n = 15$, MSUS $n = 13$). Data are presented as mean ± SEM, $^*P < 0.05$. Part of the data were published in Gapp *et al.* (2014a).

RESULTS

Effects of MSUS on Coping Behaviors in Aversive Conditions

We used a light–dark box to assess behavioral response in mildly aversive conditions in males exposed to MSUS (F1) and their male offspring (F2) (Supplementary Figure S1). On this task, an animal can escape from a brightly lit aversive compartment of a box by moving to a dark compartment. F1 MSUS males spent more time in the bright compartment of the box than controls ($t(35) = -2.14$, $P < 0.05$; Supplementary Figure S2a). Similarly, the F2 MSUS males also spent more time in the bright compartment ($t(41.61) = -3$, $P < 0.01$; Figure 1a), indicating reduced escape response suggestive of a form of resistance to aversive conditions in both generations. This effect was independent from the latency to first enter the dark compartment since it was similar in all groups (fathers: $t(34) = 0.023$, $P > 0.05$, Supplementary Figure S2b; offspring, $t(63) = 0.03$, $P > 0.05$; Figure 1b). We confirmed this effect by testing the males in even more aversive conditions using an active avoidance task. On this task, an animal is exposed to a foot-shock that can be terminated by a nose-poke into a hole. F2 MSUS males had shorter latency to nose-poke ($F(1,23) = 8.50$, $P \leqslant 0.01$) and thus received fewer foot-shocks than control offspring across time ($F(1,26) = 7.74$, $P \leqslant 0.01$) (Figure 1c and d), suggesting more active coping response. This was not due to improved learning since F2 MSUS and control males had similar nose-poke performance on a FR paradigm, a reward-based task for which a water-deprived animal learns to nose-poke into one hole to obtain a water reward in a neighboring hole ($F(1,26) = 1.33$, $P > 0.05$) (Supplementary Figure S3). These results suggest transmission of altered coping behaviors across generations.

Reversibility of Altered Responses in Aversive Conditions

EE has been reported to reverse some of the behavioral symptoms induced by chronic stress (Hutchinson *et al*, 2012) or early life adversity (Leshem and Schulkin, 2012). Using EE, we examined if the effects of MSUS on coping behaviors can be reversed (Supplementary Figure S4). We exposed F1 MSUS males to EE from weaning till adulthood. After EE, F1 males were bred to control females to generate an F2 generation, and both F1 and F2 males were tested on the light–dark box. When compared with F1 control males raised in standard cages, F1 control males raised in EE spent more time in the lit compartment of the box ($t(52) = -0.9$, $P < 0.05$) and had shorter latency to enter the dark compartment ($t(52) = -2.11$; $P < 0.05$, Supplementary Figure S2), suggesting an effect of EE on response to adverse conditions. F1 MSUS males exposed to EE had similar performance to control males exposed to EE; they spent a comparable amount of time in the lit compartment ($t(15) = -0.78$, $P > 0.05$) and had similar latency to first enter the dark compartment ($t(16) = -0.38$), $P > 0.05$) (Supplementary Figure S2c and d). However, their F2 male offspring spent a similar amount of time in the lit compartment ($t(20.85) = 1.94$, $P > 0.05$) and had lower latency ($t(17.89) = 2.33$, $P < 0.05$) than the F2 offspring of control EE fathers (Figure 2a and b), suggesting a reversal of the altered response to an adverse environment by paternal EE. Decreased latency may indicate more spontaneous escape behavior in the F2 offspring of MSUS males exposed

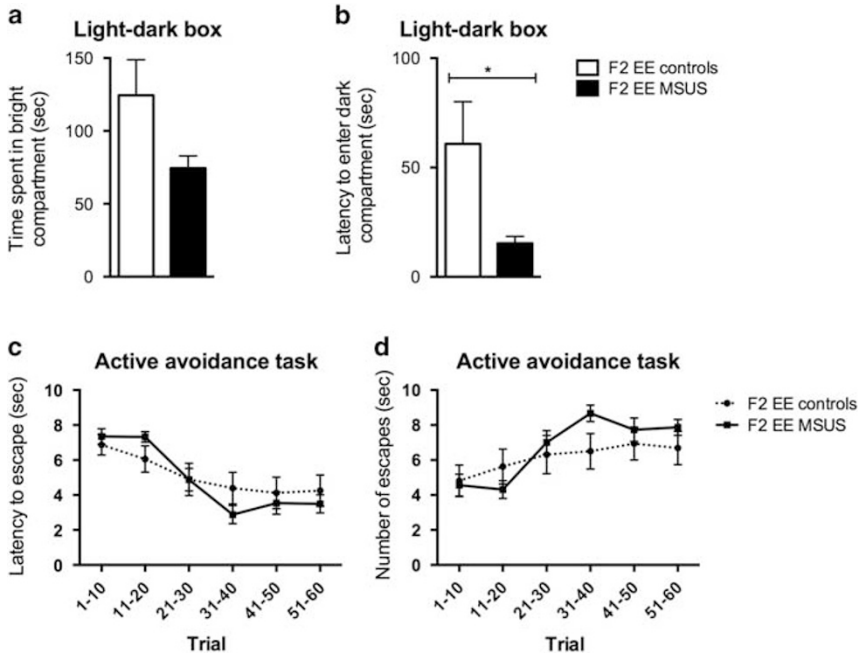

Figure 2 Aversive behavior in the offspring of males exposed to maternal separation combined with unpredictable maternal stress (MSUS) in postnatal life and environmental enrichment (EE) in adulthood. Performance in a light–dark box shown by (a) time spent in the bright compartment (EE: Controls $n=19$, MSUS $n=15$; group effect of housing $F(1,97) = 6.09$, $P<0.05$)) and (b) latency to first enter the dark compartment (EE: Controls $n=18$, MSUS $n=16$; interaction between housing and treatment $F(1,97) = 7.56$, $P<0.01$)). (c) Latency to escape a foot-shock (group effect of treatment $F(1,53)=4.72$, $P<0.05$; interaction between treatment and housing $F(1,53)=3.07$, $P=0.09$) and (d) total number of foot-shocks (group effect of treatment $F(1,51)=7.22$, $P\leqslant 0.1$; interaction between treatment and housing $F(1,53)=3.41$, $P=0.07$) on the active avoidance task (EE: Controls $n=16$, MSUS $n=13$). Data are presented as mean ± SEM. $^*P<0.05$.

to EE. This is consistent with a decrease in the time spent in the bright compartment, suggesting a reduction in overall escape behavior in the F2 offspring of MSUS exposed to EE and a reversal of the effect of paternal MSUS exposed to standard housing. Similarly, responses on the active avoidance task were reversed by paternal EE in F2 MSUS males. The F2 offspring of MSUS fathers exposed to EE had similar latency to escape ($F(1,27)=0.09$, $P>0.05$) and number of escapes ($F(1,28)=0.42$, $P>0.05$) to the F2 offspring of control fathers exposed to EE (Figure 2c and d).

Effects of MSUS and EE on *GR* Expression and Promoter Methylation

Since GR has been implicated in stress responses and coping behaviors, we examined if its expression is altered by MSUS in the hippocampus, a key brain structure implicated in the shut down of stress responses (de Kloet *et al*, 2005b). Using qRT-PCR, we observed a significant increase in GR expression in F2 MSUS males when compared with control males ($t(11)=-2.7$, $P<0.05$). This increase was not observed in the offspring of F1 MSUS males exposed to EE ($t(12)=-0.84$, $P>0.05$) (Figure 3a), indicating a correction of *GR* expression by paternal EE. Hippocampal *GR* expression was also increased in F1 MSUS fathers ($t(13)=-2.45$, $P<0.05$) and this increase was as well reversed by EE (Supplementary Figure S5). To assess the functional relevance of changes in *GR* expression, we conducted proteome-wide analyses of proteins in F1 and F2 hippocampus of control and MSUS males using a labeling method based on iTRAQ and mass spectrometry. This unbiased method allows the rigorous identification and quantification of multiple proteins at the same time in the same samples. The results of these proteomic analyses revealed that 17 proteins known to be glucocorticoid-response element targets (Datson *et al*, 2011; Gray *et al*, 2014; Polman *et al*, 2012) were altered by at least 1.3-fold in the hippocampus of adult MSUS males compared with adult control males. These changes seem to be a functional consequence of altered GR mRNA levels. They also significantly add to the functional relevance of the MSUS model by identifying the affected targets. The list of altered proteins and affected targets is provided in Table 1.

Because *GR* gene is known to be regulated by DNA methylation (Weaver *et al*, 2004), we examined if DNA methylation is altered by MSUS. We used quantitative bisulfite pyrosequencing to measure DNA methylation in exons 1–7 of the *GR* gene, a region that contains transcription factor binding sites important for transcriptional regulation (Mueller and Bale, 2008; Figure 3c). In F2 MSUS offspring, exon 1–7 methylation was significantly decreased at CpG3 ($t(26)=2.33$, $P<0.05$) and CpG7 ($t(26)=2.19$, $P<0.05$) (Figure 3b). Importantly, this decrease was reversed by paternal EE (CpG3: $t(10)=2.17$, $P>0.05$; CpG7: $t(10)=-0.97$, $P>0.05$; Figure 3b), indicating a correction of aberrant DNA methylation by EE in MSUS offspring. Since this effect accompanies a normalization of gene expression, it suggests functional relevance for *GR* expression. Consistently in F1 MSUS males, DNA methylation at the *GR* gene is lower and *GR* expression is increased in the hippocampus (CpG2: $t(9)=3.43$, $P<0{,}01$; CpG3: $t\ (9)=2.31$, $P<0{,}05$; Supplementary Figure S6). We next

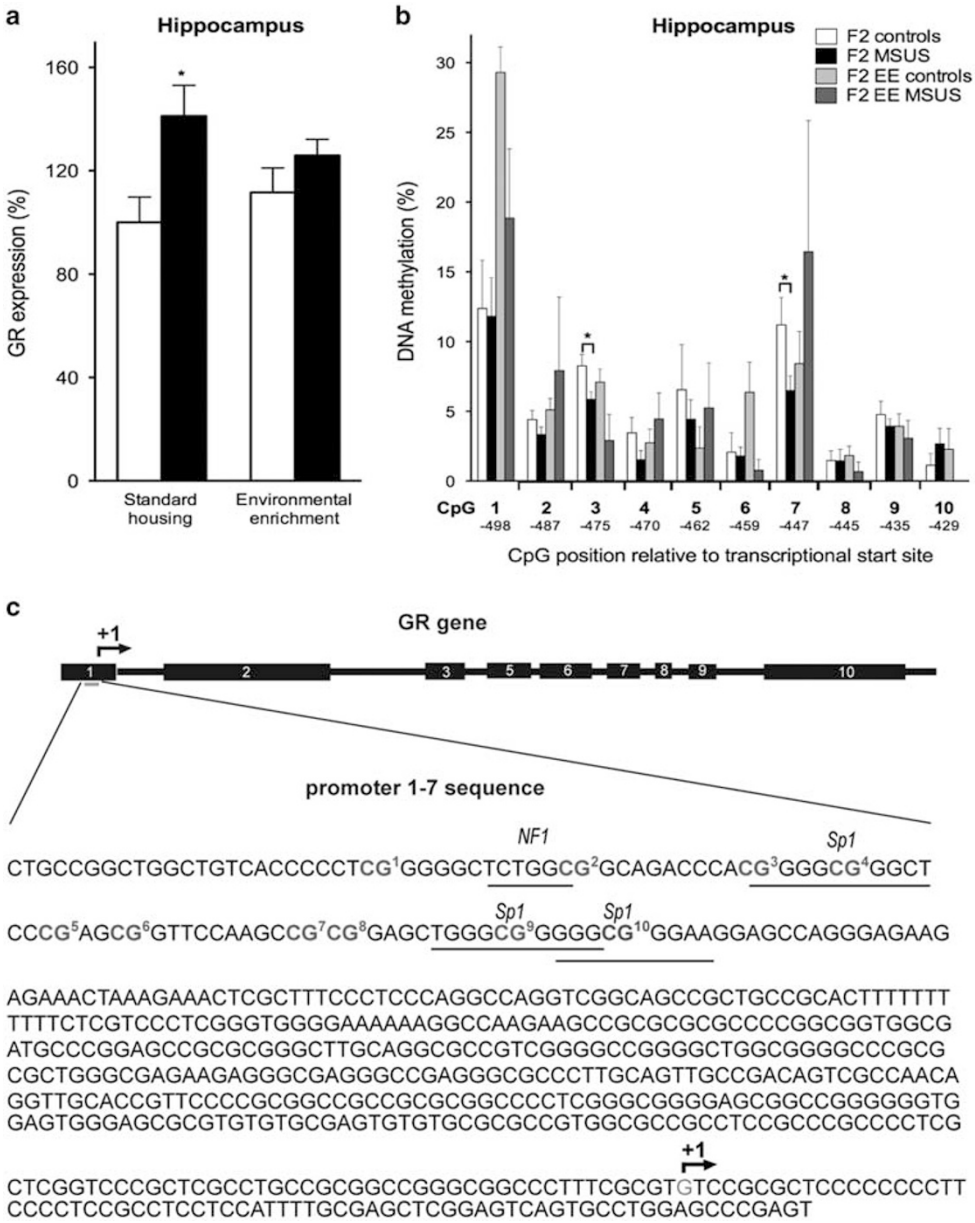

Figure 3 Molecular analysis in the F2 offspring of F1 fathers exposed to maternal separation combined with unpredictable maternal stress (MSUS) and environmental enrichment (EE). (a) Glucocorticoid receptor (*GR*) gene expression measured by quantitative reverse transcriptase-PCR (Standard housing (SH): Controls $n=6$, MSUS $n=6$; EE: Controls $n=7$, MSUS $n=5$; group effect of treatment $F(1,21)=7.74$, $P\leqslant 0.01$). (b) DNA methylation at *GR* exon 1–7 promoter in the hippocampus assessed by bisulfite pyrosequencing (SH: Controls $n=14$, MSUS $n=14$; EE: Controls $n=7$, MSUS $n=5$; group effect of treatment at CpG2 $F(1,20.8)=0.05$ and at CpG3: $F(1,61)=7.94$, $P\leqslant 0.01$; a tendency for group effect of housing at CpG3: $F(1,23.31)=3.03$ and an interaction between housing and treatment at CpG4: $F(1;43,9)=3.97$, $P\leqslant 0.05$). (c) Schematic representation of the *GR* gene and the untranslated exon 1–7 promoter sequence examined for DNA methylation by pyrosequencing assays including the transcription start site (+1). Predicted transcription factor binding sites are underlined and in italic. Exons are shown as black boxes. Numbers represent individual CpG sites analyzed for DNA methylation (CpG 1–10). Data are presented as mean ± SEM, $^{*}P<0.05$ *t*-test comparison.

examined if the altered DNA methylation is passed to the offspring from fathers by analyzing DNA methylation in sperm cells of F1 males. DNA methylation was significantly decreased at CpG3 (t (19.4) = 2.77, $P<0.05$, $P<0.05$) and CpG7 (t (20) = 2.3, $P<0.05$), consistent with hypomethylation at the same CpG in the offspring's hippocampus. We further detected a decrease at CpG1 ($t(14.03)=3.47$, $P<0.01$), CpG8 ($t(14.07)=3.48$, $P<0.01$), and CpG9 ($t(14.15)=3.5$, $P<0.01$) (Figure 4). Importantly, these effects did not persist in the sperm of F1 fathers that had been exposed to EE, suggesting a correction of the effects of MSUS by EE at the germline level (CpG1 $t(8.03)=-19$, $P>0.05$, $P<0.05$; CpG3 $t((8.02)=-2.09$, $P>0.05$, $P<0.05$; CpG7 $t(9)=-2.26$, $P=0.05$, CpG8 $t((-8.01)=-2$; $P>0.05$, $P>0.05$; CpG9 $t(8.03)=-2.12$, $P>0.05$; Figure 4).

DNA methylation induced by MSUS in sperm may be maintained after fertilization and during development, and thus be present in different tissues in the offspring. We examined this question by assessing DNA methylation in another brain region, the prefrontal cortex, in adult

Table 1 Proteome-Wide Protein Expression in the Hippocampus of F1 MSUS Males and Their F2 Male Offspring

Generation	Protein accession number	Gene name	Regulation	Validated GR target	% Control	Generation	Protein accession number	Gene name	Regulation	Validated GR target	% Control
F1 fathers	Q69Z96	Atp13a1	Down		420.0	F2 offspring	O08908	Pik3r2	Down		63.6
F1 fathers	O55142	Rpl35a	Down		65.9	F2 offspring	O55142	Rpl35a	Down		42.8
F1 fathers	O35343	Kpna4	Down		62.1	F2 offspring	Q3UKW2	Calm1	Down		44.5
F1 fathers	Q9Z239	Fxyd1	Down		63.8	F2 offspring	Q9Z239	Fxyd1	Down		62.4
F1 fathers	B9EKC3	Arhgap5	Down		61.0	F2 offspring	Q62178	Sema4a	Down		47.5
F1 fathers	P21995	Emb	Down		64.9	F2 offspring	Q71M36	Cspg5	Down		63.5
F1 fathers	O35874	Slc1a4	Down	X	69.5	F2 offspring	Q9DCZ4	Apoo	Down		61.6
F1 fathers	Q80U23	Snph	Down	X	74.2	F2 offspring	O08599	Stxbp1	Down		64.0
F1 fathers	Q8JZK9	Hmgcs1	Down		70.4	F2 offspring	P35802	Gpm6a	Down		61.5
F1 fathers	Q6GQX8	Gm15800	Down		70.9	F2 offspring	P55096	Abcd3	Down		66.2
F1 fathers	Q7TN99	Cpeb3	Down		73.6	F2 offspring	Q8BGZ4	Cdc23	Down		60.1
F1 fathers	Q9JMG7	Hdgfrp3	Down	X	70.2	F2 offspring	Q8K386	Rab15	Down		60.7
F1 fathers	P62137	Ppp1ca	Down		76.2	F2 offspring	Q9CQE1	Nipsnap3b	Down		57.0
F1 fathers	Q9QZB1	Rgs20	Down		68.8	F2 offspring	A2BI30	RP24-297H17.3	Down		64.2
F1 fathers	Q08460	Kcnma1	Down	X	71.2	F2 offspring	Q8BH80	Vapb	Down	X	66.0
F1 fathers	Q69ZP3	Pnkd	Down	X	72.9	F2 offspring	Q9CPU4	Mgst3	Down		52.7
F1 fathers	P39053	Dnm1	Down		74.7	F2 offspring	P07724	Alb	Down		65.7
F1 fathers	Q99JR1	Sfxn1	Down		76.0	F2 offspring	Q91W34	C16orf58	Down		64.5
F1 fathers	Q9CW46	Raver1	Down		69.8	F2 offspring	B9EKN8	Tnik	Down		66.2
F1 fathers	Q9D125	Mrps25	Down		74.2	F2 offspring	Q9CPP6	Ndufa5	Down		56.1
F1 fathers	B1ARB9	Ddx5	Down		76.2	F2 offspring	Q9CZB4	Apool	Down	X	30.4
F1 fathers	Q9D6D0	Slc25a27	Down		76.8	F2 offspring	Q8K356	Ly6h	Down		58.2
F1 fathers	Q91W39	Ncoa5	Down		75.9	F2 offspring	O70503	Hsd17b12	Down		58.4
F1 fathers	Q8VCE2	Gpn1	Down		72.5	F2 offspring	Q3U0D9	Hace1	Down		66.0
F1 fathers	Q9D6U8	Fam162a	Down		75.6	F2 offspring	Q811S7	Ubp1	Down		53.8
F1 fathers	Q8R1N4	Nudcd3	Down		70.1	F2 offspring	Q9ES97	Rtn3	Down		65.6
F1 fathers	Q9DC51	Gnai3	Down		72.4	F2 offspring	Q9Z2W9	Gria3	Down		63.4
F1 fathers	Q52KF3	Spire1	Up	X	165.8	F2 offspring	Q78IK2	Usmg5	Down		60.4
F1 fathers	Q8C0W0	Tmsb15l	Up		154.7	F2 offspring	Q62277	Syp	Down		67.3
F1 fathers	P14069	S100a6	Up	X	151.2	F2 offspring	P52479	Usp10	Down	X	71.0
F1 fathers	Q9JIG8	Praf2	Up		169.2	F2 offspring	Q9JHU9	Isyna1	Down		76.6
F1 fathers	P59279	Rab2b	Up		168.0	F2 offspring	P21278	Gna11	Down		71.1
F1 fathers	Q8BRF7	Scfd1	Up		150.4	F2 offspring	Q8BGZ1	Hpcal4	Down		74.6
F1 fathers	O88935	Syn1	Up		181.2	F2 offspring	Q91VC3	Eif4a3	Down		73.2
F1 fathers	A2AFJ1	Rbbp7	Up		136.4	F2 offspring	Q921W4	Cryz1l	Down		66.7
F1 fathers	Q62048	Pea15	Up		131.6	F2 offspring	Q924M7	Mpi	Down		75.8
F1 fathers	Q3U409	Ablim2	Up		136.6	F2 offspring	P63082	Atp6v0c	Down		70.6
F1 fathers	B1ASW5	Trappc1	Up		141.7	F2 offspring	Q7JCY6	mt-Nd4	Down		68.6
F1 fathers	Q9D3D9	Atp5d	Up		136.2	F2 offspring	P56564	Slc1a3	Down		72.8
F1 fathers	Q99LY9	Ndufs5	Up		132.3	F2 offspring	Q9D9M2	Usp12	Down		70.7
F1 fathers	Q99M87	Dnaja3	Up	X	148.6	F2 offspring	Q9CR57	Rpl14	Down		72.1
F1 fathers	P0C7L0	Wipf3	Up	X	136.3	F2 offspring	P39688	Fyn	Down		72.7
F1 fathers	B2RS21	Nudt19	Up		131.6	F2 offspring	Q60930	Vdac2	Down	X	71.6
F1 fathers	P31786	Dbi	Up		135.3	F2 offspring	Q8CIP4	Mark4	Down		75.1
F1 fathers	Q3TY60	Fam131b	Up		138.2	F2 offspring	Q9CQN6	Tmem14c	Down		73.7
F1 fathers	Q9JJU8	Sh3bgrl	Up		131.8	F2 offspring	Q3TCD4	Eci2	Down		69.8
F1 fathers	O08529	Capn2	Up		131.4	F2 offspring	Q8BG32	Psmd11	Down	X	74.0
F1 fathers	O35449	Prrt1	Up	X	138.4	F2 offspring	Q9Z1X2	Ptdss2	Down		66.8
F1 fathers	P62274	Rps29	Up	X	132.9	F2 offspring	O70579	Slc25a17	Up		152.1
F1 fathers	P62996	Tra2b	Up		140.3	F2 offspring	Q2PFD7	Psd3	Up		154.1
F1 fathers	Q68ED7	Crtc1	Up		142.6	F2 offspring	Q8VHH5	Agap3	Up		138.4
F1 fathers	Q8BK30	Ndufv3	Up		147.1	F2 offspring	Q60972	Rbbp4	Up		138.1
F1 fathers	Q9D1Q6	Erp44	Up		133.0	F2 offspring	Q8BGD9	Eif4b	Up		131.1
F1 fathers	Q3U7M5	Abhd4	Up		142.1	F2 offspring	B1AWD9	Clta	Up		133.2
F1 fathers	Q9DBB8	Dhdh	Up		141.7	F2 offspring	Q9JLT4	Txnrd2	Up		141.5
F1 fathers	B1AQE6	Ikzf3	Up		144.0	F2 offspring	Q7TN29	Smap2	Up		146.8
F1 fathers	Q3UH27	Asap2	Up		139.6	F2 offspring	Q52KR3	Prune2	Up		133.5
F1 fathers	P16390	Kcna3	Up	X	131.7	F2 offspring	Q61166	Mapre1	Up		144.9
F1 fathers	Q52KR3	Prune2	Up		133.3	F2 offspring	Q6R891	Ppp1r9b	Up		130.5
						F2 offspring	A2AN47	Golga2	Up		131.7
						F2 offspring	P97930	Dtymk	Up		140.0

Proteins with differential expression ($P < 0.05$) in MSUS and controls and a minimal fold change of 1.3 measured by iTRAQ followed by mass spectrometry. Proteins with significanlty different level ($p \leqslant 0.05$) of at least 1.3-fold. Proteins significantly dysregulated in both F1 and F2 generations are in grey tint.

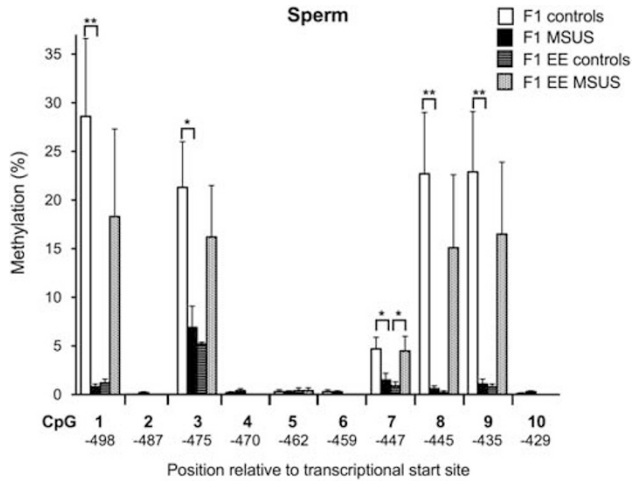

Figure 4 DNA methylation in sperm from males exposed to maternal separation combined with unpredictable maternal stress (MSUS) and environmental enrichment (EE). GR exon 1–7 promoter methylation in sperm cells of fathers assessed by bisulfite pyrosequencing (SH: Controls $n=15$, MSUS $n=10$; EE: Controls $n=11$, MSUS $n=9$; interaction between treatment and housing at CpG1 $F(1,6043.71)=13.19$, $P<0.01$; CpG3 $F(1,0.39)=14$, $P<0.01$; CpG7 $F(1,173.42)=14.18$, $P<0.01$; CpG8 $F(1,3832.58)=13.59$, $P<0.01$; CpG9 $F(1,3900.57)=14.28$, $P<0.01$). Data are presented as mean ± SEM, $*P\leqslant 0.05$, $**P\leqslant 0.01$, *t*-test comparison.

offspring. Bisulfite pyrosequencing analyses in prefrontal cortex revealed that DNA methylation was not altered at CpG3 ($t(14)=-0.17$; Supplementary Figure S7). Instead, DNA methylation was decreased at CpG2 ($t(13.77)=2.64$, $P<0.05$, $P<0.05$) and CpG7 ($t(14)=1.94$; $P<0.1$) in the F2 offspring of MSUS males exposed to standard housing (Supplementary Figure S7). These effects were not observed in the F2 offspring of MSUS males exposed to EE at CpG2 ($t(13)=-1.01$; $P>0.05$), and were reversed at CpG7 ($t(13)=-2.26$; $P<0.05$) (Supplementary Figure S7). Further, MSUS increased DNA methylation in the prefrontal cortex at CpG4, in both the F2 offspring of standard housed and EE fathers (Supplementary Figure S7). The observed changes differ from those in the hippocampus, suggesting that changes in DNA methylation in sperm cells are not systematically passed to all cells in the offspring. Region/tissue-specific mechanisms likely mediate the transgenerational effects and the interplay of early life stress and housing conditions.

DISCUSSION

The present results show that the offspring of males exposed to traumatic stress during early postnatal life are better able to appraise and respond to adversity when adult. At a molecular level, this effect is associated with GR and involves increased *GR* expression and decreased DNA methylation of *GR* promoter in the hippocampus. Strikingly, the behavioral changes are reversible and behavior in the offspring is normalized when fathers are exposed to EE in adulthood. This is accompanied by a correction of DNA hypomethylation at some CpGs of the *GR* gene in the sperm of fathers and the hippocampus of the offspring.

In humans and primates, traumatic experiences often increase the risk for psychopathology but in some conditions, they can lead to resilience (Lyons and Parker, 2007), a form of active coping when faced with a challenging environment, later in life (Zozulya *et al*, 2008). Similarly in rodents, successful adaptation after exposure to chronic stress in early life has been observed, and has been associated with changes in the HPA axis (Franklin *et al*, 2011; Gapp *et al*, 2014b; Uchida *et al*, 2011). Thus, the response of individuals exposed to negative experiences in early life can be pathological or adaptive depending on the context, a concept theorized by the match/mismatch hypothesis (Daskalakis *et al*, 2012). The present results are in line with this hypothesis because they show that the offspring of males exposed to early trauma can cope better with a challenging situation, similarly to that experienced by their father. Further, they extend previous observations that MSUS animals have improved behavioral flexibility in aversive conditions (Gapp *et al*, 2014b), suggesting that exposure to early trauma can lead to various forms of resilience.

Active avoidance is a form of adaptive behavior that involves, in part, *GR* expression in the hippocampus (Korte, 2001). One of the known modes of regulation of *GR* expression implicates DNA methylation at exons 1–7 of the *GR* gene. *GR* expression is known to be modulated by maternal care (Weaver *et al*, 2004). Here, we show that *GR* expression is also responsive to other factors, in particular traumatic stress in postnatal life and EE in adulthood. *GR* expression has been reported to be sex-specific at baseline (Elakovic *et al*, 2011) and has been linked to sex-specific responses in changing environments (Lin *et al*, 2011). Interestingly, while *GR* expression is increased in MSUS males, it is not altered in MSUS females (Gapp *et al*, 2014b), indicating sex-specific alteration by traumatic stress. The mechanisms for male-specific effects are not known but could involve DNA methylation. For instance, several algorithms predict the existence of a binding site for the transcription factor SP1 at CpG3, a CpG affected by MSUS (Tsunoda and Takagi, 1999). SP1 is known to regulate *GR* expression in humans (Suehiro *et al*, 2004) and other mammals (Zou *et al*, 2013), and act by dimerizing with the sex hormone receptor ER (Klinge, 2001). SP1–ER interactions could differentially interfere with the hippocampal response to stress causing sex-specific differences in expression.

Our data on DNA methylation in F1 sperm and F2 hippocampus suggest the implication of this epigenetic mark in the regulation of *GR* gene expression and the inheritance of its alterations in our model. At some CpGs, DNA methylation was reduced in the sperm of F1 fathers and the hippocampus of their offspring. The alterations were corrected in sperm by paternal EE and DNA methylation was normal at these sites in the offspring. However, at this stage, it is not possible to determine whether and which CpG(s) directly control the regulation of *GR* expression, and it is likely that other mechanisms are also implicated. Our proteomic data indeed suggest that several proteins involved in gene transcription, protein translation, peptide and amide biosynthesis relevant to *GR* expression may also contribute since they were differentially expressed in the hippocampus of F1 or F2 MSUS males compared with controls. In particular, the nuclear receptor coactivator 5 (Ncoa5),

a putative binding partner of steroid receptors like GR that can act as a transcriptional coactivator or repressor, was found to be downregulated. Further, the human growth and transformation-dependent protein (HGTDP or Fam162a), involved in GR localization upon activation to the nucleus, are downregulated. In contrast, DNAJ3a a direct interaction partner of GR (Hedman *et al*, 2006) is upregulated. DNAJ3a stimulates Hsp70 leading to GR inactivation (Kirschke *et al*, 2014) and impacting GR autoregulation. These results therefore suggest that, in the brain, *GR* expression is likely controlled by different mechanisms that may complement DNA methylation.

EE is a non-invasive and natural paradigm that increases the level of sensory, cognitive, and motor stimulation and promotes brain plasticity. It has been used to restore hippocampal integrity after chronic stress (Hutchinson *et al*, 2012) and may have some potential to counteract symptoms of psychopathologies and neurodegenerative diseases (Hannan, 2014; Nithianantharajah and Hannan, 2006). Our findings demonstrate that EE can reverse the effects of early traumatic stress. The reversal might involve adaptive mechanisms resulting from the mismatch between favorable EE conditions and adverse conditions in early life. These mechanisms may also involve epigenetic regulation of *GR* gene in the hippocampus, consistent with the idea that chronic stress and EE recruit similar pathways within the hippocampus (Hutchinson *et al*, 2012). Additional molecular pathways may also be involved. Besides regulating GR via DNA methylation, EE might regulate GR at the transcriptional level. Since GR is an auto- and cross-regulated gene, initial hypermethylation observed in the sperm of environmentally enriched MSUS males might first lead to decreased *GR* expression. This might release the negative autofeedback by GR and thereby normalize GR levels while at the same time, affect the recruitment of epigenetic enzymes such as DNA methyl transferases/demethylases that ultimately decrease DNA methylation.

The mechanisms underlying germline-dependent epigenetic inheritance remain not fully understood but have been proposed to implicate one or more epigenetic marks including DNA methylation, histone/protamine post-translational modifications and RNA (Gapp *et al*, 2014c). Here, we show that DNA methylation likely contributes to the transmission of alterations in *GR* expression in response to environmental exposures such as MSUS and EE. This suggests that DNA methylation is modifiable at some CpGs during postnatal life and adulthood. The data also show that while some changes in sperm methylome are present in the offspring, some are not maintained in the offspring, suggesting corrective mechanisms. These mechanisms are likely tissue- and cell-specific. The causal involvement of DNA methylation in epigenetic inheritance remains however to be demonstrated. But this is complicated by the large inter-individual variability of the methylome in sperm, which is a strong confounding factor (Laurentino *et al*, 2016). Our previous work demonstrated the involvement of sperm RNA in the transmission of some behavioral and metabolic traits in the MSUS model (Gapp *et al*, 2014a). Sperm non-coding RNAs like microRNAs could also be involved in the transgenerational regulation of *GR* gene but to date, no miRNA that can target GR have been identified in MSUS sperm (Gapp *et al*, 2014a).

In summary, our data provide new evidence that some forms of coping can be induced by trauma exposure in early life and be transmitted to the offspring. They also newly demonstrate that behavior and the epigenome in the germline and the brain are sensitive to the environment and can be modified across life. These findings have potential implications for the design of diagnostic and therapeutic approaches for trauma-related psychopathologies.

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