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RESEARCH ARTICLE

Transgenerational inheritance of behavioral and metabolic effects of paternal exposure to traumatic stress in early postnatal life: evidence in the 4th generation

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Abstract

In the past decades, evidence supporting the transmission of acquired traits across generations has reshaped the field of genetics and the understanding of disease susceptibility. In humans, pioneer studies showed that exposure to famine, endocrine disruptors or trauma can affect descendants, and has led to a paradigm shift in thinking about heredity. Studies in humans have however been limited by the low number of successive generations, the different conditions that can be examined, and the lack of mechanistic insight they can provide. Animal models have been instrumental to circumvent these limitations and allowed studies on the mechanisms of inheritance of environmentally induced traits across generations in controlled and reproducible settings. However, most models available today are only intergenerational and do not demonstrate transmission beyond the direct offspring of exposed individuals. Here, we report transgenerational transmission of behavioral and metabolic phenotypes up to the 4th generation in a mouse model of paternal postnatal trauma (MSUS). Based on large animal numbers (up to 124 per group) from several independent breedings conducted 10 years apart by different experimenters, we show that depressive-like behaviors are transmitted to the offspring until the third generation, and risk-taking and glucose dysregulation until the fourth generation via males. The symptoms are consistent and reproducible, and persist with similar severity across generations. These results provide strong evidence that adverse conditions in early postnatal life can have transgenerational effects, and highlight the validity of MSUS as a solid model of transgenerational epigenetic inheritance.

Key words: transgenerational; epigenetic inheritance; mouse model; early-life trauma; behavior; 3rd and 4th generation

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Introduction

The concept of transgenerational epigenetic inheritance implies that epigenetic signatures induced by exposure can be maintained across generations and may be responsible for the manifestation of phenotypes in parents and their offspring. This stands in contrast to the Mendelian model of inheritance by which genetic factors are the sole hereditary vectors of trait variation. Transgenerational epigenetic inheritance has major implications for disease etiology in humans and helps explain complex conditions such as neuropsychiatric, metabolic and immunological disorders whose heritability cannot be explained only by genetic factors [1]. Although the concept initially met reservation due to conceptual limitations such as the fact that the epigenome is reprogrammed in developing germ cells and the early embryo, evidence has accumulated to indicate that environmentally induced epigenetic alterations and phenotypes can indeed be transmitted across generations in mammals (for recent reviews see Refs. 2–5).

Epidemiological studies in humans have implicated grandparental and parental environmental conditions such as nutrient availability, exposure to endocrine disruptors or traumatic experiences in disease susceptibility in descendants [6, 7]. In mammals, rodent models of coat color linked to the agouti^{vy} locus and DNA methylation [8] or exposure to the endocrine disruptor vinclozolin [9], have shown altered phenotypes that are stably transmitted to non-exposed offspring. Other exposure models, including various dietary regimes [10–18], environmental toxins [19–26], postnatal trauma [27], prenatal glucocorticoids [28], prenatal immune activation [29], psychotropic medication [30] or olfactory stimulation [31], have linked exposure to altered traits in non-exposed offspring. However today, most models are intergenerational and exhibit traits that are transmitted only to the direct offspring of exposed individuals but not to further generations. Moreover, most models use exposure until breeding, and thus have effects in the progeny that may be due to the acute exposure of sperm at the time of breeding that may not persist beyond exposure, which is a limitation.

To gain understanding of the mechanisms of transgenerational epigenetic inheritance, models with transmission up to the 3rd generation or possibly to further generations, are needed. Our lab has developed a mouse model of early postnatal trauma based on unpredictable maternal separation combined with unpredictable maternal stress (MSUS) in which multiple effects were documented in the offspring up to three generations. Mice exposed to MSUS and their offspring have increased risk-taking behaviors [27, 32–37], depressive-like symptoms [27, 32–37], altered social recognition [38], memory deficits [35] and insulin/glucose dysregulation [34]. They also show stress resilience [38] and improved behavioral flexibility [33] in some conditions. The symptoms are transmitted through both males and females [27, 32].

Because this model is robust and epigenetic changes have been detected in the male germline and in tissues in the offspring [27, 34] (female germline not tested), we examined whether symptoms are also present in the 4th generation produced from the patriline. The results show that both behavioral and metabolic symptoms induced by MSUS are present in mice from the 4th generation, indicating that MSUS is a solid and reproducible transgenerational model of early life adversity.

Results

MSUS Paradigm

The MSUS paradigm was designed to mimic in mice, exposure to traumatic experiences during childhood in humans. MSUS is based on the combination of adverse conditions subjected to young mouse pups during early postnatal life and to their mother during the same period (Fig. 1). The paradigm consists of separating mouse pups (F1) from their mother (F0) unpredictably each day for 3 hours from postnatal day 1 (PND1) to PND14 (Fig. 1A). In this paradigm, unpredictability is critical because it avoids that mothers predict separation and compensate for their absence by providing more maternal care before separation. Instead, it leads to an overall decreased and disorganized maternal care, especially between PND1 and PND7 [27]. Further to separation, dams are also exposed to an additional stressor unpredictably (anytime during the 3 hours of separation) (Fig. 1B). Here again, unpredictability is important as it increases the severity of the stressor, and the combination of unpredictable maternal separation with such unpredictable maternal stress was shown to induce stronger behavioral phenotypes in the offspring than separation alone [32]. Breeding was conducted until the 4th generation by mating adult males at each generation (F1, F2 and F3) with naïve primiparous control females (Fig. 1C).

Transgenerational Effects of MSUS are Robust and Reproducible, and Observed Until the 4th Generation

The MSUS paradigm has been repeatedly demonstrated to cause behavioral and metabolic alterations in adulthood, not only in directly exposed mice (F1) but also in their (unexposed) offspring (F2) [27, 32, 34–36, 38] and grand-offspring (F3) [27, 38]. We consolidated these findings by obtaining different cohorts of mice from several MSUS breedings across several years. The mice were tested on different tasks by different experimenters and the data were pooled to reach large *n* (Supplementary Table S1). When tested on an elevated plus maze, F1 and F2 MSUS males spent more time on the open arms of the maze (Fig. 2A), confirming transmission of reduced aversion to open space from father to offspring. The grand-offspring (F3) did not show this phenotype. However, mice from F1, F2 and F3 generations had decreased latency to first enter an open arm (Fig. 2B), suggesting increased risk-taking behavior that is transgenerationally transmitted to F3. Further to the elevated plus maze, F3 mice were also tested on a forced swim test to assess passive coping, a trait characteristic of depressive-like behaviors. Consistent with that observed previously [27], F3 MSUS males spent more time floating than controls (Fig. 3A), confirming that depressive-like symptoms are reproducible in the 3rd generation.

While transgenerational transmission across 3 generations has been reported twice with different behavioral tests in MSUS mice [27, 38], transmission to the 4th generation has not yet been examined. We produced a 4th generation of MSUS and control mice by breeding F3 males to control females and generated 2 batches of mice, 10 years apart (2007 and 2017; Supplementary Table S2) then tested the male offspring when adult (different experimenters). When tested on the elevated plus maze, F4 males from both batches spent significantly more time in the open arms of the maze and had significantly shorter latency to first enter an open arm, similar to F1, F2 and F3 males (Fig. 3B). Total distance covered was not changed, as expected (Supplementary Fig. S1A). Then, when tested on the forced swim test, F4 MSUS males (Batch 2 only, Batch 1 not tested) spent a similar amount of time floating to controls (Fig. 3C), suggesting no depressive-like symptoms thus no apparent transmission of this trait beyond F3.

To examine if these effects affect both sexes, we also tested F4 MSUS and control females. Similar to males, F4 MSUS females spent more time in the open arms and had shorter latency to first enter an open arm on the elevated plus maze compared to F4

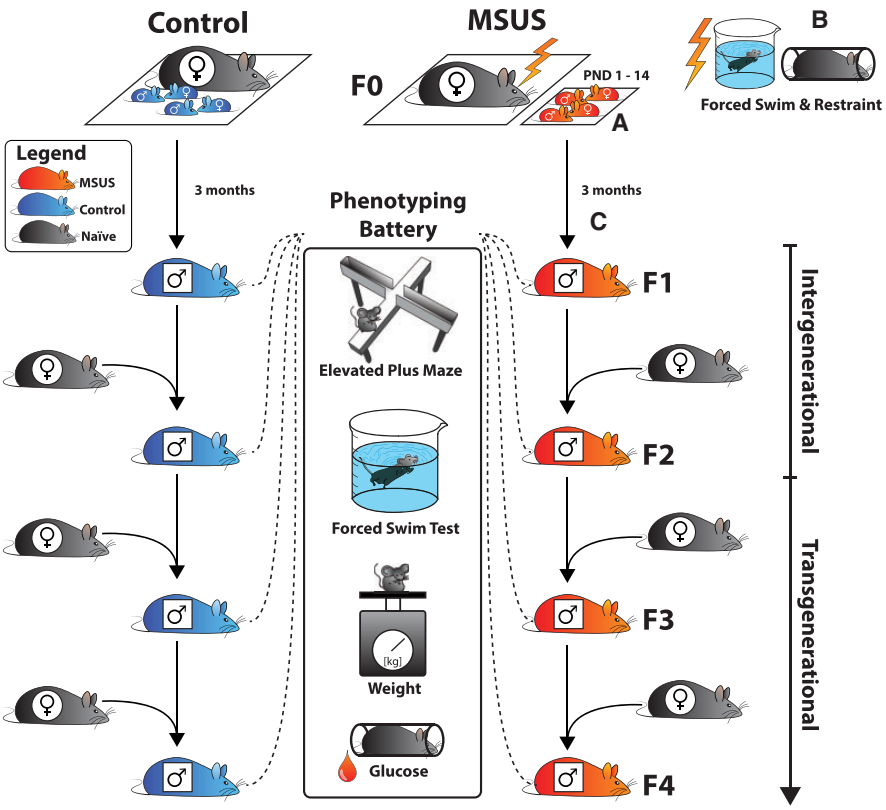

Figure 1: MSUS paradigm. MSUS consists of (**A**) separating mouse pups (F1) from their mother (F0, naïve primiparous control females mated with naïve males) daily for 3 hours per day at an unpredictable time during the 12 hours active cycle, starting 1 day after birth (postnatal day 1, PND1) until PND14. (**B**) During separation, dams are exposed to an additional unpredictable stressor by being subjected to either, a forced swim in 18°C water for 5 minutes or a 20-minute physical restraint in a tube, anytime (unpredictably) during the 3 hours. From PND15, mice are left undisturbed with their mother until PND21 (no further MSUS), are then weaned at PND21 and raised normally until adulthood (**C**). Control litters are raised normally (left). Males used to generate the pups are removed from the breeding cage shortly after mating thus, fathers never encounter their offspring and do not contribute to their rearing. When adult (3–8 months of age), F1 males are paired with naïve primiparous control females to sire the F2 generation, then F2 and F3 males are bred with naïve primiparous control females to generate an F3 and F4 offspring, respectively. Males from each generation are tested on the elevated plus maze, forced swim test, weight measurements and glucose response after physical restraint. MSUS is applied only to F1 mice, mice from F2, F3 and F4 generations are not exposed to any manipulation. Phenotypes transmitted from father to offspring are intergenerational, phenotypes that persist from father to offspring then grand-offspring or great grand-offspring are transgenerational

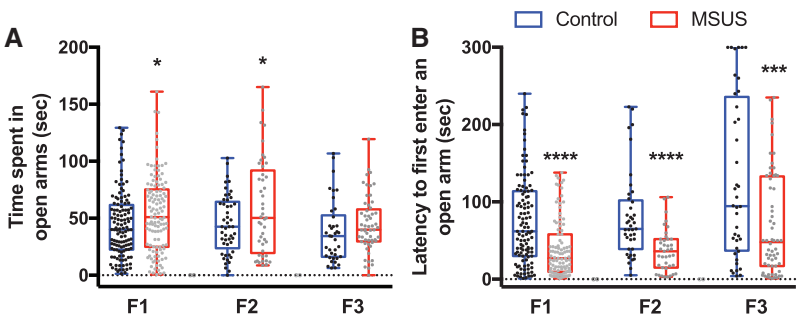

Figure 2: persistent behavioral effects of MSUS across 3 generations on the elevated plus maze. MSUS treatment (**A**) increases the amount of time spent on the open arms of an elevated plus maze in F1 and F2 mice but not F3 mice (F1 control $n = 124$, MSUS $n = 118$, $t_{240} = 2.26$ $P = 0.025$; F2 control $n = 49$, MSUS $n = 45$, $t_{92} = 2.096$ $P = 0.039$; F3 control $n = 38$, MSUS $n = 57$, $t_{93} = 1.244$ $P = 0.217$) and (**B**) decreases the latency to first enter an open arm in F1, F2 and F3 mice (F1 control $n = 111$, MSUS $n = 101$, $t_{210} = 5.298$ $P < 0.0001$; F2 control $n = 41$, MSUS $n = 39$, $t_{78} = 4.353$ $P < 0.0001$; F3 control $n = 40$, MSUS $n = 59$, $t_{97} = 3.51$ $P = 0.0007$). Data represent median ± whiskers. Reported n represents data after outlier removal using the ROUT test at Q = 5%. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$

controls (Fig. 4B), but had normal locomotor activity (Supplementary Fig. S1B). F4 females spent a similar amount of time floating as control females in a forced swim test (Fig. 4A), suggesting no depressive-like symptoms, similar to F4 MSUS males. These findings establish that the effects of paternal MSUS persist across generations, some until the F3 and F4 generations. Furthermore, they highlight risk-taking behavior as a highly consistent trait that similarly affects males and females up to the 4th generation.

Metabolic Effects of MSUS are Transmitted Transgenerationally

MSUS has been shown to dysregulate glucose and insulin levels in F1 mice and their offspring [34]. We examined if these

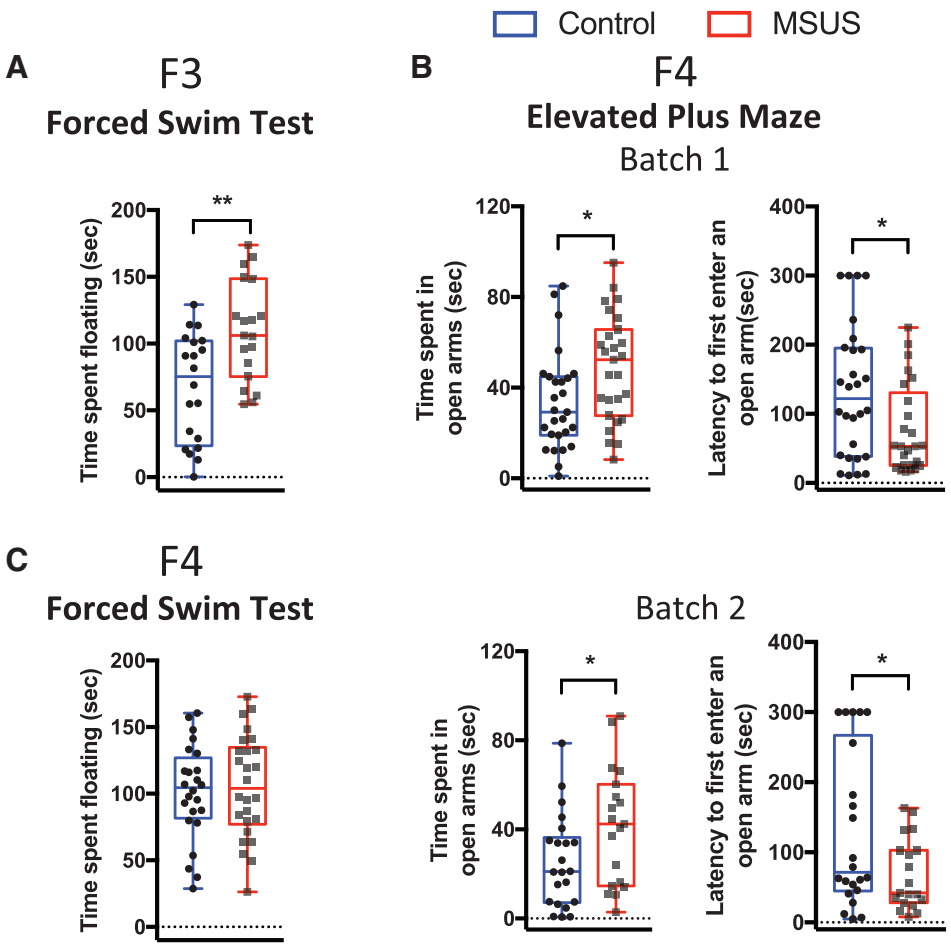

Figure 3: reproducible behavioral alterations by MSUS in F3 and F4 generations. Depressive-like symptoms shown by increased time spent floating on a forced swim test in MSUS males from (**A**) F3 generation but not from (**C**) F4 generation (F3: control $n = 20$, MSUS $n = 19$ $t_{37} = 3.37$ $P = 0.0018$; F4: Batch 2 control $n = 24$, MSUS $n = 26$ $t_{48} = 0.424$ $P = 0.6732$). In (**B**), separate batches of F4 males (Batches 1 and 2) were tested on the elevated plus maze. Time spent on the open arms and latency to first enter an open arm are similarly altered in both batches. (Batch 1 for time spent on open arms: control $n = 22$, MSUS $n = 19$, $t_{52} = 2.49$ $P = 0.0161$; Batch 1 for latency to enter an open arm: control $n = 22$, MSUS $n = 19$, $t_{51} = 2.432$ $P = 0.019$; Batch 2 for spent time on open arms: control $n = 27$, MSUS $n = 27$, $t_{39} = 2.159$ $P = 0.037$; Batch 2 latency to first enter an open arm: control $n = 28$, MSUS $n = 25$, $t_{39} = 2.209$ $P = 0.033$). Data represent median ± whiskers. Reported n represents data after outlier removal using the ROUT test at $Q = 5\%$. $^{*}P < 0.05$, $^{**}P < 0.01$

symptoms are also present in mice from the F3 and F4 generations. In both F3 and F4 MSUS males, there was a trend for increased glucose at baseline compared to control males (Fig. 5A), unlike F2 MSUS males which had decreased glucose levels [34]. However, during a physical restraint challenge, the mounting of glucose response was modestly but significantly attenuated in F4 MSUS males compared to controls, similar to that observed in F2 MSUS males [34] (Fig. 5B). This effect was not observed in F3 MSUS males (Supplementary Fig. S2). Interestingly in F4 MSUS males, body weight was lower than controls at PND21 but slightly increased in adulthood (Fig. 5C), suggesting a rebound response. This response may be due to increased food consumption as indicated by higher food intake in MSUS mice (Fig. 5D), but does not result from an inherently larger body size since tail length was normal in MSUS mice (Fig. 5E). Body weight and food intake in F4 females was unaffected by MSUS (Supplementary Fig. S3).

Discussion

This study provides evidence that exposure to traumatic stress in early postnatal life in mice induces several behavioral alterations that are transmitted across several successive generations. While increased risk-taking and glucose dysregulation affect mice up to the 4th generation, depressive-like behaviors affect F3 but not F4 MSUS males. This indicates that risk-taking is a robust trait that perpetuates in descendants, suggesting that it may be more penetrant than other traits. This may be because, although disadvantageous in some conditions, it may be beneficial in challenging situations and provide a form of active coping advantage. In contrast, passive coping associated with behavioral despair as observed on the forced swim test was not expressed by F4 MSUS mice. This however does not mean that this trait has disappeared, since we observed in the past that some F2 MSUS males did not show any depressive-like symptoms (were asymptomatic) but were still able to transmit this trait to their offspring [27]. The manifestation of both risk-taking and depressive-like behaviors in MSUS mice is interesting because, together with antisocial behaviors observed in F3 males [38], these traits are typical of a common psychiatric disorder, borderline personality disorder (BPD), which has a lifetime prevalence of 6.4% in the population [39]. BPD is a severe

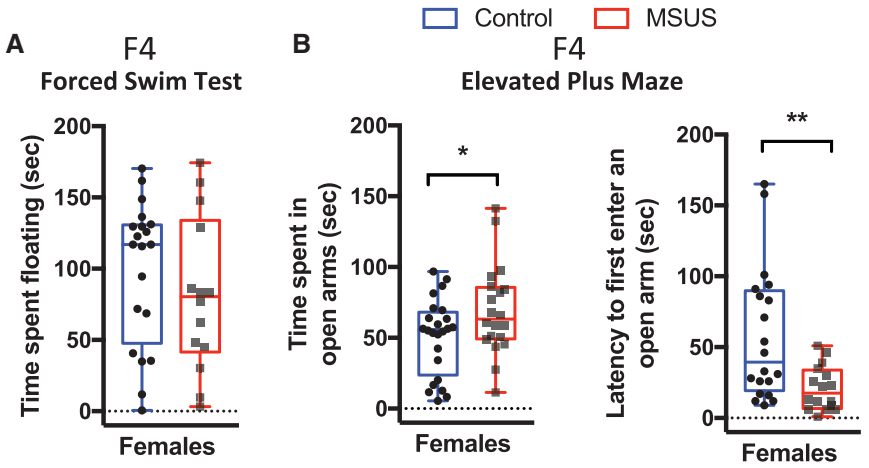

Figure 4: behavioral phenotypes in F4 female progeny. (**A**) F4 MSUS females do not significantly differ from control females in time spent floating during the forced swim test (control $n = 20$, MSUS $n = 14$, $t_{32} = 0.918$ $P = 0.366$). (**B**) Time spent in the open arms of the elevated plus maze was increased (control $n = 24$, MSUS $n = 20$, $t_{42} = 2.09$ $P = 0.043$), while latency to first enter an open arm was decreased in F4 MSUS females (control $n = 20$, MSUS $n = 16$, $t_{34} = 3.01$ $P = 0.005$). Data represent median ± whiskers. Reported n represents data after outlier removal using the ROUT test at Q = 5%. *$P < 0.05$, **$P < 0.01$

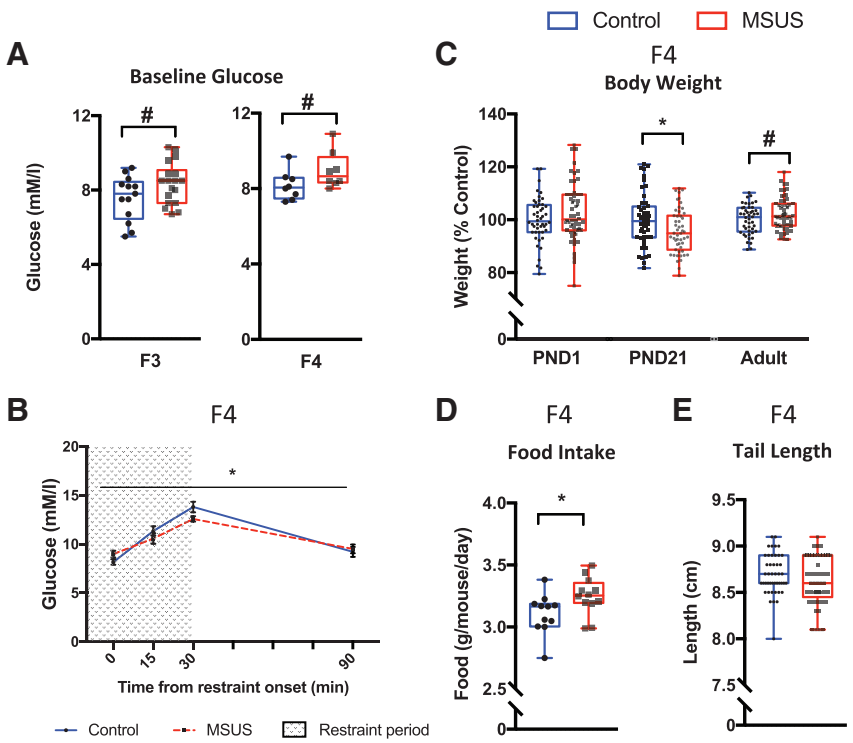

Figure 5: transgenerational effects of MSUS treatment on glucose level. (**A**) Baseline glucose was measured in whole blood following tail prick in F3 (left) and F4 males (right) (F3 control $n = 13$, MSUS $n = 18$, $t_{29} = 1.891$ $P = 0.069$; F4 control $n = 8$, MSUS $n = 8$, $t_{14} = 1.84$ $P = 0.087$). Continuing from (A), glucose concentrations in F4 blood (**B**) was measured at 15-minute intervals during a 30-minute physical restraint challenge, and 60 minutes after release from the restraint tube (control $n = 8$, MSUS $n = 8$, for interaction $F_{3, 42} = 2.99$ $P = 0.042$). (**C**) Body weight of F4 males was measured at PND1, PND21 and in adulthood (PND1: control $n = 48$, MSUS $n = 52$, $t_{99} = 1.29$ $P = 0.199$; PND21: control $n = 46$, MSUS $n = 50$, $t_{94} = 2.27$ $P = 0.025$; adult: control $n = 46$, MSUS $n = 51$, $t_{95} = 1.86$ $P = 0.065$). (**D**) Food intake (control $n = 11$, MSUS $n = 12$, $t_{21} = 2.185$ $P = 0.04$; n represents number of cages) and (**E**) tail length were measured in adult mice (control $n = 46$, MSUS $n = 51$, $t_{92} = 1.38$ $P = 0.172$). Data represent median ± whiskers, except for (B) which represent mean ± s.e.m. Reported n represents data after outlier removal. #$P < 0.1$, *$P < 0.05$

condition characterized by impulsive behaviors leading to risky and potentially life-threatening conduct [40], emotional liability including depressed mood [40] and impaired social functioning [41, 42]. MSUS mice also have memory deficits [35] and stress-induced analgesia (unpublished data), which are other prominent BPD symptoms [43, 44]. Notably, BPD has a strong heritability component that cannot be explained by genetic factors alone [45], and instead, the risk to develop the disorder has been associated with adverse childhood experiences [46, 47]. Traumatic experiences in humans are known to result in maladaptive coping strategies [48] and in increased risk-taking behavior when occurring in childhood [49]. The

environmental etiology of BPD and its known heritability suggest that it likely involves epigenetic factors, possibly in the germline. While in humans, germline-dependent inheritance is difficult to prove and cannot easily be distinguished from social and rearing factors e.g. being raised by a parent with a psychiatric illness can predispose a child to psychiatric illness [50], germline-dependent inheritance implicating sperm RNA has been causally demonstrated in our MSUS model [34].

Further to behavioral deficits, MSUS also causes metabolic alterations across generations. Metabolic symptoms have been reported in humans exposed to trauma, and metabolic syndrome can develop in response to prolonged stress [51] and in people suffering from BPD [52] and post-traumatic stress disorder [53]. Notably, the effects of MSUS on metabolism are expressed differently across generations. While in F2 MSUS males, baseline glucose is downregulated [34], it is slightly upregulated in F3 and F4 MSUS mice. This is in contrast to behavioral traits which are similarly expressed across generations but is a phenomenon already observed in other transgenerational models [9, 17]. Differential expression of phenotypes across generations has been reported in other models of transgenerational epigenetic inheritance [54].

In addition to male phenotypes, we also extend the previously reported transgenerational effects to MSUS females [27] until the fourth generation. Behavioral differences in F4 MSUS females are directly comparable to F4 MSUS males, suggesting similar trait penetrance through the patriline in females. However, this is not the case for metabolic phenotypes, suggesting different mechanisms of transmission depending on the phenotype. Regarding potential mechanisms of transmission, our past work demonstrated a causal role for sperm RNA in the transfer of phenotypes [34], and correlated changes in DNA methylation in sperm with transgenerational phenotypes [27], suggesting that several epigenetic factors are likely implicated. Others factors or mechanisms may also be involved [55, 56].

A distinct and unique feature of the MSUS paradigm is that it is postnatal, which provides a significant advantage over prenatal models because it avoids interference with gestational developmental processes and epigenetic reprogramming occurring during embryogenesis [2]. MSUS exposure is also brief and limited to a specific time window between PND1 and PND14, allowing for easier identification of the cells affected during that time, in particular in developing gonads which have only a limited number of different cell types at this stage. Most other models have exposure extending from before conception throughout embryogenesis and postnatal development until adulthood [10, 13–15, 31], making interpretation more difficult as each developmental stage is likely to be affected, producing cumulated effects. Furthermore, in models with adult exposure extending until breeding, the effects can result from acute factor(s) in gonads, supporting cells or seminal fluid at the time of conception, and may not just implicate germ cells. With MSUS, breeding occurs many months after exposure, eliminating any acute changes and selecting for effects that persist until adulthood. This persistence suggests that spermatogonial cells may be affected. Another unique advantage of MSUS is that it does not involve any drug, chemical, nutritional insult or invasive manipulation and relies on “natural” aspects of childhood mistreatment such as neglect, attachment disruption and abuse. Other physiological parameters like altered maternal milk composition or lower body temperature due to separation may also be implicated.

The use of a battery of behavioral tasks including the elevated plus maze, forced swim test and in previous studies, the open field test, light-dark box, emergence test, fear conditioning, social interaction task, operant conditioning, object recognition and social defeat, and of several parameters on some of the tests e.g. latency to enter and time spent in open arms on the elevated plus maze, generated a comprehensive and thorough behavioral profiling of MSUS mice across generations. After conducting MSUS treatment in 30 independent experiments since 2001 with up to 40 breeding pairs each time, this study identifies the elevated plus maze and forced swim as reliable tests to validate the effects of MSUS across generations. The effects observed on these tasks are robust, consistent and highly reproducible. MSUS is currently one of the few available mouse models of transgenerational epigenetic inheritance with transmission up to the 4th generation, a depth of inheritance previously demonstrated in rodents with prenatal stress [57], toxicants [9, 24], drugs [30, 58] or genetic mutation (*Mtrr* hypomorphic) [59]. Studies of the mechanisms of transgenerational inheritance are expected to have important implications for public health in the future.

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Supplementary data

Supplementary data are available at *EnvEpig* online.

Conflict of interest statement. None declared.

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