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Nandini Doshi April 9, 2017

Physiological and Behavioral Imprints of Parental Olfactory Experience

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Abstract

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Epidemiological studies suggest that severe stress or trauma has the ability to influence the development of neuropsychiatric disorders in the descendants of those directly exposed to the adverse event. Studies using animals corroborate these findings and allow us to study these influences. Previous laboratory investigations have confirmed that paternal exposure to stressful stimuli is associated with altered stress-regulation in subsequent generations. Missing from this discussion is how and why this phenomenon occurs. Olfaction provides a behavioral and structural framework that allows us to follow how a stressor perceived by one generation can leave imprints on the behavior, physiology, and neurobiology of future generations. Previous research has shown that exposing F0 mice to olfactory fear conditioning (odor + mild foot-shock) results in an increased sensitivity and an enhanced neuroanatomy for that odor in male F1 offspring. However, it remains unknown how that F1 generation behaves and physiologically responds when they encounter that paternally conditioned odor. In the present study, we examined behavioral and physiological responsiveness of F1 animals when they encounter the paternally salient odor. We subjected male mice to olfactory fear conditioning, odor presentations alone, or allowed them to remain in their home cages and then tested the behavior of their offspring in response to a presentation of the F0 conditioned odor (Acetophenone, Ace). Additionally, we measured corticosterone levels of the F1 generations in response to an Ace presentation. Male, but not female, F1 offspring of Ace-conditioned animals exhibited significantly greater freezing and a blunted corticosterone response to an exposure of Ace relative to the F1 offspring of Ace-exposed animals. Moreover, the offspring of olfactory conditioned males were also subjected to a weak conditioning paradigm to the paternally salient

odor and showed a decreased threshold for learning to the F0-conditioned odor but no difference in stress regulation after a presentation of that odor one day after the weak-conditioning paradigm. These results suggest that parental experiences may leave imprints in their offspring via corticosterone action to bias their behavior.

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INTRODUCTION

Psychiatric Disorders and Risk Factors

Community epidemiological surveys approximate that nearly 30% of the U.S. adult population suffers from one or more DSM psychiatric disorders, including anxiety disorders, mood disorders, impulse and control disorders, and substance use disorders, over a 12-month period (Kessler et al., 1994). Within this subset, anxiety disorders, such as panic disorder, generalized anxiety disorder, obsessive-compulsive disorder, and post-traumatic stress disorder, consist of the most prevalent (18.1%) class of DSM psychiatric disorders (Kessler et al., 2005). Although psychiatric disorders are considered to be one of the leading agents of disability, their morbidity and resulting mortality rates are expected to increase over the near future (Ferrari et al., 2014). Only 41.1% of those affected by DSM psychiatric disorders receive some form of treatment (Wang et al., 2005). Evidently, more effective and widespread preventive and therapeutic options must be developed, but in order to establish such strategies, further investigation of the risk factors and causes of psychiatric disorders must occur.

Studies suggest the trajectories and phenotypes of neuropsychiatric illnesses involve interactions between genes and environment, with the environmental component of profound influence (Kendler 1995; Sullivan et al., 2013; Klengel et al., 2016). While such environmental factors, including traumatic or stressful events, can exert a robust impact on the course of a disease when they occur during critical periods of brain development, such as *in utero* or early childhood, an increasing amount of evidence also indicates that severely stressful life experiences can affect offspring as well (Klengel et al., 2016). For instance, various clinical observations have indicated that the offspring of stress-exposed parents are more susceptible to behavioral, cognitive, and physical complications (Bowers and Yehuda, 2016).

Intergenerational Transmission of Stress: Human Studies

Research studies focusing on intergenerational human cohorts exposed to traumatic historical events suggest that stressful life experiences have the potential to not only impact the populations directly exposed, but they also have the ability to affect the health and behavior of descendants. An important example of such intergenerational influences comes from the Dutch Hunger Winter during World War II. Pregnant mothers exposed to the stress of starvation gave birth to children (*in utero* during the famine) who had significantly higher risks of developing mental health diseases later in life (De Rooij et al., 2006). Likewise, a study investigating intergenerational effects of the Holocaust revealed an association between Post-Traumatic Stress Disorder (PTSD) in Holocaust survivors and the increased likelihood for their adult offspring to subsequently develop PTSD, even though those offspring were born after World War II (Yehuda et al., 1998). Along with the increased incidence of PTSD, offspring of Holocaust survivors expressed lower self-esteem and higher anxiety levels (Gangi et al., 2009). It has also been indicated that pregnant women who developed PTSD in response to trauma during the 9/11 World Trade Center attacks later reported their infants as exhibiting significantly greater distress to novel stimuli (Brand et al., 2006).

While a variety of clinical observations suggest the ability for ancestral trauma to leave imprints on the mental health of descendants, a more recent focus is the study of the neuroendocrine correlates of intergenerational transmission of stress. The hypothalamic-pituitary-adrenal (HPA) axis can be profoundly affected by environmental manipulations such that parental experiences may likely influence disease trajectories related to HPA axis function in offspring (Rodgers et al., 2013; Bowers and Yehuda, 2016). Utilizing the Dutch Famine Birth Cohort Study as a model, adults who were *in utero* during the famine exhibited blunted cortisol

reactivity in response to acute psychological stress (De Rooij, 2013). Similarly, while studying the intergenerational effects of PTSD, Yehuda and colleagues observed significantly lower cortisol levels in babies of pregnant mothers who developed PTSD in response to the World Trade Center attacks (Yehuda et al., 2005). They additionally detected decreased cortisol levels in the adult offspring of Holocaust survivors diagnosed with PTSD (Yehuda et al., 2000). Ultimately, these studies suggest that stressful environmental perturbations may result in a blunted cortisol response to stress in future generations.

Intergenerational Transmission of Stress: Animal Studies

Animal studies have further corroborated evidence from human studies suggesting the impact of parental stress on subsequent generations. For instance, a study by Dietz et al., (2011) subjected adult male mice, referred to as the F0 generation, to either a chronic social defeat paradigm, where they were exposed to a novel aggressive male mouse for ten minutes per day for ten days, or housed with another mouse without the presence of an aggressive mouse. Both groups of mice were then mated with a naïve female for ten days. Their adult male and female offspring, also referred to as the F1 generation, were subsequently subjected to a variety of behavioral tests that indicated that parental exposure to stressors like social defeat elicits heightened depressive- and anxiety-like behaviors in offspring. Likewise, Rodgers and colleagues (Rodgers et al., 2013) subjected male mice to a chronic variable stress paradigm for six weeks throughout either puberty or adulthood: F0 mice were exposed to stressors from thirty-six hours of constant light to a fifteen-minute restraint to multiple cage changes. After mating them with a naïve female and then removing the male mice from the cages before the birth of their F1 offspring, Rodgers and colleagues subjected the adult F1 generation to fifteen-minutes

of restraint stress and then measured their consequent corticosterone levels. The male and female F1 offspring of sires subjected to chronic stress throughout puberty or adulthood exhibited significantly lower corticosterone levels after the restraint. Both studies provide further evidence for the ability of parental—and more specifically, paternal—stress exposures to influence descendants. However, despite the variety of evidence supporting the intergenerational transmission of stress, little is known about how and why this phenomenon occurs. Also, the relevant neural circuitry in offspring that are affected by parental stress paradigms as broad as chronic variable stress and social defeat have not yet been thoroughly established. Therefore, in order to investigate these outstanding questions, a more focused framework must be utilized.

Olfaction as a Framework to Study Intergenerational Transmission of Stress (Fig. 1)

Olfaction provides a distinct behavioral and structural framework that allows us to follow the imprints of a stressor across generations. Each olfactory sensory neuron (OSN) expresses one specific odorant receptor (OR) gene, and axons of OSNs expressing the same odorant receptor assemble to form glomeruli in the olfactory bulb such that each glomerulus processes the sensory input relevant to a specific odorant receptor (Redolfi and Lodovichi, 2015). Mitral cell dendrites form synapses with OSN axons within a glomerulus and then innervate different cortical and subcortical regions including the piriform cortex and the amygdala (Leinwand and Chalasani, 2011). Given the ability to employ specific odor-odorant receptor pairs and that the neuroanatomy of the olfactory system has been well-established (Jones et al., 2008; DeMaria and Ngai, 2010; Leinwand and Chalasani, 2011), we can utilize olfaction as a model to investigate the circuit-level modifications and their biological causes that occur in future generations due to

parental exposure to stress. However, before we study these factors, we must first investigate and establish the behavioral and physiological implications of olfactory stress on descendants.

In effort to determine whether olfaction compliments existing models of intergenerational stress, Dias and Ressler (2014) subjected adult male F0 mice to olfactory fear conditioning using either acetophenone or propanol odor exposures paired with mild foot-shocks. They then mated these F0 mice with sexually inexperienced, odor-naïve females and removed the males from the mating pairs before the birth of the F1 generation in order to prevent any interaction between the conditioned fathers and their offspring. Upon reaching two to three months of age, the odor-naive F1 generation was subjected to an odor-potentiated startle (OPS) paradigm, which involves presenting a mouse with a startle, an odor, which for this study consisted of the paternally-conditioned odor, and that odor paired with a startle to calculate an OPS score as a measure of olfactory sensitivity. The F1 offspring of olfactory fear conditioned males exhibited an enhanced sensitivity, or ability to detect the odor at lower concentrations, to the paternally conditioned odor. Nonetheless, no study to our knowledge has utilized the well-defined olfactory framework to examine how the F1 generation actually behaves upon encountering the stimulus that paternal ancestors found aversive. Moreover, it still remains unknown how F0 olfactory experience leaves imprints on the physiology of their direct offspring.

To address these outstanding questions, we built on the study referenced above and utilized olfactory fear conditioning to determine how olfactory stress influences the behavior, physiology, and cognition of subsequent generations. We proposed that subjecting F0 generation males to an olfactory fear conditioning paradigm would result in an enhanced sensitivity to the paternally-conditioned odor in their F1 generation offspring. Moreover, we also hypothesized that the F1 generation would exhibit a blunted corticosterone response consequent to an exposure

to the paternally conditioned odor. Lastly, we assessed how paternal olfactory fear conditioning and the resulting altered sensitivity in descendants may impact their learning. To do so, we subjected the F1 offspring to a weak olfactory conditioning paradigm consisting of a single odor-shock pairing and proposed that the F0 fear conditioning would promote a decreased threshold for learning to the same odor in the F1 generation.

MATERIALS AND METHODS

Animals

All experiments were performed with two- to three-month-old sexually- and odor-inexperienced adult male mice. Mice were housed on a twelve-hour light/dark cycle in cages of five or fewer animals per cage with *ad libitum* access to food and water in a temperature-controlled vivarium. All treatment and testing protocols were administered during the light half of the cycle. Both M71-LacZ and M71-GFP strains of mice used in the experiments were bred in the Yerkes Neuroscience animal facility. All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with the guidelines set by the US National Institutes of Health.

F0 Fear Conditioning (Fig. 2; Fig. 3)

Two- to three-month-old M71-LacZ transgenic male mice were divided into three groups: the home cage control group (F0-Home, n=2), the odor only group (F0-Exposed, n=6) and the odor plus foot-shock group (F0-Trained, n=7). The F0-Exposed and F0-Trained groups were habituated inside the SR-LAB Startle Response System (San Diego Instruments) chamber for 5 minutes each day for two consecutive days prior to treatment. The F0-Trained group then received five 10-second acetophenone (10% acetophenone in propylene glycol) presentations co-terminating with a 0.25-second 0.4 mA foot-shock over fifteen minutes on three consecutive days. In contrast, the F0-Exposed group received five 10-second acetophenone presentations with no foot-shocks over fifteen minutes on three consecutive days, and the F0-Home group remained in their chambers in the vivarium. Ten days later, each mouse from all three groups

was mated with naïve M71-GFP transgenic female mice. Thirteen days later, these matings were separated in order to prevent any interaction between the males and their offspring.

Testing for intergenerational olfactory sensitivity in F1 generation (Fig. 2; Fig. 4)

The F1 generation mice were weaned from their mothers at 25 days of age. F1 generation adult mice (two to three months of age) were habituated inside the Mouse Habitest Chambers (Coulbourn Instruments) to the testing conditions (smooth black floors, lights on, cleaned with quatricide, air and vacuum on) for three minutes each on two consecutive days. They were then exposed to seven minutes of air followed by one 30-second exposure to acetophenone (F1-Exposed-Sensitivity, Males: n=10, Females: n=13; F1-Trained-Sensitivity, Males: n=15, Females: n=16), and their freezing prior and in response to the odor presentation was recorded and analyzed using FreezeFrame software (Actimetrics).

Testing for altered threshold for learning in the F1 generation (Fig. 2; Fig. 5)

F1 generation adult mice were habituated inside the Mouse Habitest Chambers (Coulbourn Instruments) to the training condition (shocker floors, house lights on, cleaned with ethanol, air and vacuum on: Context A) for three minutes each on day one. On the next day, they were habituated inside the same chambers to the testing condition (smooth black floors, IR lights on, cleaned with quatricide, air and vacuum on: Context B) for three minutes each. On Day 3, F1-Home-Learning (Males: n=12; Females: n=10) and F1-Trained-Learning (Males: n=12; Females: n=15) were exposed to seven minutes of air followed by a 20-second acetophenone odor co-terminating with a 0.5 second 0.4 mA foot-shock in Context A. Utilizing only one odor foot-shock pairing presents as a weak conditioning paradigm, so the animals generally do not

demonstrate much learning to this conditioning. Their learning was measured through the following: one day after training, these animals were presented with seven minutes of air followed by a 30-second exposure to the conditioned odor in Context B, and their freezing behavior prior and in response to the odor presentation was recorded and analyzed utilizing the FreezeFrame software.

Tissue Collection (Fig. 4 and 5)

All F1 mice were sacrificed thirty minutes following the collection of their freezing responses to odor presentations. Each mouse's trunk blood was collected, stored on ice, and subsequently processed for serum one hour after blood collection through centrifuging each blood sample for fifteen minutes at 3500 RPM at -4^{o}C. The serum samples were stored in 30 µL aliquots at -80^{o}C.

Corticosterone Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples from F1 generation mice (F1-Trained-Sensitivity Males, n=5; F1-Trained-Sensitivity Females, n=5; F1-Exposed-Sensitivity Males, n=5; F1-Exposed-Sensitivity Females, n=5; F1-Trained-Learning Males, n=5; F1-Trained-Learning Females, n=5; F1-Home-Learning Males, n=5; F1-Home-Learning Females, n=4) were analyzed for corticosterone levels utilizing the Corticosterone ELISA Kit (Catalog #: ADI-900-097, Enzo Life Sciences, NY) following manufacturer instructions. Serum samples were diluted 30-fold through combining 8.32 µL of serum, 6.25 µL of Steroid Displacement Reagent, and 235.5 µL of Assay Buffer. All samples wells were loaded in duplicates with 100 µL of the diluted samples. The NSB wells were loaded in duplicate with 150 µL Assay Buffer and 50 µL Corticosterone ELISA Conjugate Solution, and the 0 pg/mL wells were loaded in duplicate with 100 µL Assay Buffer.

Corticosterone standards were prepared for five concentrations (20,000 pg/mL, 4000 pg/mL, 800 pg/mL, 160 pg/mL, and 32 pg/mL) utilizing the provided provided Corticosterone Standard Solution, and standard wells were loaded in duplicates with 100 µL of each standard. Fifty microliters of the provided Corticosterone ELISA Conjugate Solution were added to all wells except for the TA and blank wells, and 50 µL of the provided Corticosterone ELISA Antibody Solution was added to all wells except for the NSB, TA, and blank wells. The ELISA plate was covered and incubated on a shaker for two hours at room temperature.

The contents of the wells were removed and washed three times with 400 µL of Wash Buffer. Five microliters of Corticosterone ELISA Conjugate Solution was added to the TA wells and 200 µL of the provided p-NPP substrate was added to all wells. The plate was subsequently subjected to a one-hour incubation.

Fifty microliters of the provided Stop Solution was added to each well, and the optical densities of the wells were read utilizing the Multiskan FC Microplate Photometer plate reader and SkanIt Software (ThermoFisher Scientific) at 405 nm and 620 nm with blank well subtractions in order to measure the corticosterone concentrations. The corticosterone concentrations values from the 620 nm reading were subtracted from those in the 405 nm reading to calculate the corrected corticosterone concentrations of each serum sample.

Statistical Analysis

Statistical significance of freezing behavior and corticosterone levels was analyzed by the Student's *t* test. Significance level was set at $p < 0.05$.

RESULTS

F0 Olfactory Experience Affects Behavior and Physiology in Subsequently Conceived Generation

Behavior (Fig. 6 and 7)

To determine how paternal olfactory fear conditioning influences the behavior of offspring, F1 generation animals were exposed to one presentation of the paternally conditioned odor, and their percent freezing prior to that odor and during that odor was measured. Analysis of their freezing behaviors indicated significantly greater freezing in male offspring of Ace conditioned males (n=15) compared to the male offspring of Ace exposed males (n=10) during the odor presentation [$t(23) = 2.257$, $p = 0.03$]. There was no significant difference in freezing behaviors between both groups before the odor presentation [$t(23) = 0.4827$, $p = 0.63$]. Although this effect was observed in the male offspring, there was no significant freezing prior to [$t(27) = 0.1672$, $p = 0.8685$] and during [$t(27) = 0.4703$, $p = 0.6419$] the odor presentation in the female F1 offspring of Ace conditioned males (n=16) relative to the female offspring of Ace exposed males (n=13).

Physiology (Fig. 8)

Thirty minutes after the odor presentation, blood was collected from all mice and processed for serum to measure F1 stress responsivity after an acetophenone presentation. A corticosterone ELISA revealed that both male and female F1 offspring of Ace-conditioned F0 males (Males: n=5, Females: n=5) exhibited significantly lower serum corticosterone levels than the male and female offspring of Ace-exposed F0 animals (Males: n=5, Females: n=5) [Males: $t(8)$, $p = 0.0439$; Females: $t(8)$, $p = 0.0256$].

F0 Olfactory Experience Results in Decreased Threshold for Learning Related to F0-Conditioned Odor in F1 Generation

Behavior (Fig. 9 and 10)

To investigate how paternal olfactory fear conditioning influences the learning threshold of their offspring, the F1 generation was subjected to a weak conditioning paradigm for the F0-conditioned odor, and twenty-four hours later, they were presented with the odor once again to measure their consequent freezing behavior. Analysis of freezing behaviors indicated that the male offspring of mice that were olfactory fear conditioned (n=12) exhibited significantly greater freezing behavior than the offspring of home cage control animals (n=12) to acetophenone on testing day [$t(23) = 2.319$, $p = 0.031$]. There was no difference in freezing prior to the acetophenone exposure [$t(22) = 0.8169$, $p = 0.4228$]. Likewise, female offspring of olfactory conditioned F0 males (n=15) exhibited significantly greater freezing behavior than the offspring of home cage control animals (n=10) to acetophenone on testing day [$t(23) = 2.546$, $p = 0.0181$]. They exhibited no significant difference in freezing behavior prior to the odor presentation [$t(23) = 0.1495$, $p = 0.8825$).

Physiology (Fig. 11)

Similar to the previous experiment, thirty minutes after the collection of freezing behavior data, blood was collected from all animals and processed for serum to measure physiological responsiveness to a weak conditioning for the paternally-conditioned odor. The results from the serum corticosterone ELISA demonstrated no significant difference in serum corticosterone levels between the male offspring of home cage control animals (n=5) and olfactory fear conditioned animals (n=5) [$t(8) = 0.9289$, $p = 0.3801$]. Likewise, the female offspring of home cage control males (n=4) and olfactory fear conditioned males (n=5) exhibited

similar corticosterone levels in response to an acetophenone presentation one day after a weak conditioning paradigm to that odor [$t(7) = 1.761$; $p = 0.1217$].

DISCUSSION

Utilizing olfactory fear conditioning as a framework allowed us to follow the influence of a specific olfactory experience on the behavior and physiology of subsequently conceived generations. Through analyzing freezing behavior, we first found that the F1 generation male offspring of olfactory conditioned F0 sires exhibited an enhanced behavioral sensitivity towards the F0 conditioned odor relative to the F1 generation male offspring of odor-exposed sires. These data replicate a previously published finding using another behavior assay and this experimental design (Dias and Ressler, 2014). However, the freezing behavior data from the female F1 descendants of olfactory conditioned F0 males revealed that they did not possess an altered behavioral sensitivity towards the F0 conditioned odor. We also found that both the male and female F1 offspring expressed a blunted corticosterone response after a single exposure to the paternally aversive odor. Lastly, we observed that the F1 generation sired by olfactory conditioned F0 males exhibited a decreased threshold for learning to the odor used to condition F0 mice, but this altered cognition was not accompanied by altered stress regulation. Both the male and female offspring of conditioned animals demonstrated similar corticosterone regulation as the F1 descendants of home cage control animals after a single presentation of the odor that F0-trained animals were conditioned to and all F1 animals were weakly trained to.

Other studies have similarly investigated the salience of a specific environmental experience on future generations. Our findings are consistent with previous findings indicating that descendants of stressed sires exhibit altered behavior, such as enhanced sensitivity or increased anxiety-like measures (Dietz et al., 2011; Dias and Ressler, 2014). Moreover, Dias and Ressler (2014) also reported that increased glomerular sizes specific to the F0 conditioned odor accompanied the enhanced sensitivity in the F1 generation and posited that this altered

neuroanatomy may facilitate the altered sensitivity observed. It is important to note that whereas the prior study measured olfactory sensitivity through odor-potentiated startle, our study utilized freezing. While our data replicate their finding, they also go further because this measure gives our findings a greater ethological significance as well since it suggests how the F1 generation will behave and respond upon encountering salient stimuli in their natural environments. Nevertheless, the Dias and Ressler (2014) study only examined the transgenerational impacts of paternal olfactory stress on the sensitivity and neuroanatomy of male descendants. The present study also includes the female F1 generation and finds no difference in behavior in response to the paternally conditioned odor. The absence of altered sensitivity in female offspring of olfactory conditioned sires may be the result from an absence of neuroanatomical changes at the level of glomeruli. Further exploration of the differences in neuroanatomy between male and female descendants of olfactory stressed males may further elucidate the causes behind the differences in sensitivity observed between both sexes.

Why this behavioral sensitivity is sex-specific is an intriguing question. Several studies have reported sex differences in intergenerational transmission of stress. For instance, paternal onset of smoking prior to the age of nine was shown to have a positive correlation with the BMI of male offspring but not female (Pembrey et al., 2006). Additionally, sex-specific effects of ancestral food supply on descendant mortality have been recorded such that paternal grandfather food supply during his slow growth period, the time before the onset of puberty when the body is more vulnerable to environmental influences, exerted a significant effect on male grandchild mortality risk ratio (Pembrey et al., 2006). Furthermore, extended paternal exposure to alcohol prior to conception of offspring is associated with a stress hyporesponsivity in male, but not female, F1 animals, and parental subjection to severe stress has also exhibited correlations with

increased risk of neurodevelopmental disorders in only male descendants (Bale and Epperson, 2015; Rompala et al., 2016). These data, along with ours, suggest that the imprints of severe stress are transmitted to male and female descendants by different mechanisms or pathways.

Although the enhanced behavioral sensitivity was observed only in the male F1 generation, both male and female offspring of olfactory conditioned F0 sires exhibited a decreased corticosterone response thirty minutes after a presentation of the paternally aversive odor. While these data support human and animal studies indicating that parental trauma or stress results in a blunted stress regulation in future generations (Yehuda et al., 2002; Yehuda et al., 2005; Van den Bergh et al., 2008; De Rooij, 2013; Rodgers et al., 2013; Danielson et al., 2015), they also suggest that the F1 generation's modified physiology and behavior resulting from parental stress are differentially mediated. It is important to note that the majority of these studies established correlations between maternal stress, either prior to conception or during gestation, and dysregulation of the stress pathway in the next generation. Prior research in rodent models indicates that exposure to stress in females can directly alter maternal care of pups, which has adverse consequences for child development (Champagne and Meaney, 2008). Thus, the blunted cortisol or corticosterone response observed in the aforementioned studies could have been socially transmitted through altered maternal care. Since the F0 sires never interacted with their offspring in the present study, we argue that the stress dysregulation was not socially transmitted, but biologically inherited. Moreover, in many of the previous studies, the stressful experience also occurred when the children found to be at risk for adverse mental, physical, and physiological outcomes were *in utero* and thus exposed to the salient event. Our study observed enhanced sensitivity in male F1 offspring followed by decreased stress regulation in male *and* female offspring of stressed sires. It is important to note that the F1 generation was

conceived after their father's environmental perturbation, so our study also provides further evidence that paternal stress can influence the neuroendocrine response of subsequently conceived offspring, which had no direct exposure to the stressful stimulus or their fathers in this study. Our findings parallel those of a study by Rodgers et al., (2013), who similarly found a blunted corticosterone response in the offspring of male mice subjected to chronic stress, and provided an epigenetic basis for the decreased HPA axis reactivity in the F1 generation via non-coding RNA in the sperm of stressed sires (Rodgers et al., 2015).

Altered cognition at the level of decreased threshold for learning to the parentally aversive stimulus might be linked to the dysregulation of the stress pathway. Chronic activation of the stress system due to sustained exposure to a stressful stimulus results in altered HPA axis regulation through adjustments in circulating levels of cortisol (Tsigos and Chrousos, 2002). Since circulating cortisol has the ability to influence gametes and the fetus, dysregulation of parental HPA axis may also mediate intergenerational transmission of stress (Graves and Eiler, 1979; Gitau et al., 1998; Matthews, 2002; Rodgers et al., 2013; Bowers and Yehuda, 2016). Our postulation that stress dysregulation in the F1 generation facilitates their decreased threshold for learning could also imply that the decreased glucocorticoid responsivity transmitted from the paternal lineage is the F0 generation's mechanism of ensuring that descendants learn faster to salient stimuli. This system would allow the F1 generation to maintain greater fitness in an environment that parents suffered adverse outcomes. On the contrary, dysregulation of the stress pathway, through decreased glucocorticoid responsivity, may indicate the ineptitude to properly adjust to environmental perturbations and therefore may suggest an increased risk for psychopathology. For instance, stress dysregulation serves as a shared characteristic of various neuropsychiatric disorders (Arborelius et al., 1999; Agid et al., 1999; Jansen et al., 2000). A

hallmark of anxiety disorders like PTSD consists of an increase in cortisol followed by a premature cessation of the cortisol response after a stressful exposure, and the relevant literature suggests that parents with such disorders transmit an increased risk for developing similar psychopathologies through disturbing the regulation of offspring HPA axis (Yehuda, 2002; Bowers and Yehuda, 2016). To further refine our findings and make more conclusive statements about the relationship between corticosterone and learning in an intergenerational context, future investigations would be well served. Moreover, it would be similarly interesting to investigate if the altered behavior, physiology, and learning observed in the F1 generation would be generalized to other odors or stressful encounters. Based on the results of the present study, we can now begin to investigate the circuit-level modifications that occur in future generations due to parental exposure to stress.

Conclusions

In summary, we utilized the framework of olfaction to explore the influence of paternal exposure to stress, prior to conception, on adult offspring. Our results indicate that male stress exposure results in a transmission of enhanced sensitivity, measured through the phenotype of behavioral freezing, towards that odor to male, but not female, offspring. Alternatively, the sex difference in sensitivity may be explained by the differences functional connectivity of the amygdala sub-regions, which are involved in processing olfactory information, observed between males and females (Alarcón et al., 2015). Paternal stress also results in a transmission of stress dysregulation to both male and female descendants when they confront a stressful experience themselves. Whereas HPA axis dysregulation, through increased cortisol or corticosterone levels, suggests a resistance to the glucocorticoid by the HPA axis, low cortisol or

corticosterone levels in subjects exposed to stressful stimuli, as observed in the present study, is considered to indicate an enhanced negative feedback inhibition to an overresponsive HPA axis (Whally et al., 1986; Yehuda et al., 1993b). Observations of greater numbers of glucocorticoid receptors in such subjects have explained the greater efficiency of the negative feedback system and thus may also explain the blunted stress response observed in the present study (Yehuda et al., 1991; Yehuda et al., 1993a). Lastly, offspring of stressed sires also exhibited altered cognition upon encountering the stimulus that their paternal ancestors found aversive. These findings may help explain why descendants of parents who experienced severely stressful or traumatic events before the conception or birth of their children have a greater risk for developing neuropsychiatric disorders themselves (Bowers and Yehuda, 2016). Ultimately, we interpret our results as providing evidence that after encountering a particularly stressful experience, parents may transmit certain phenotypes to their offspring in order to prepare them to appropriately react to similar environmental exposures.

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FIGURES

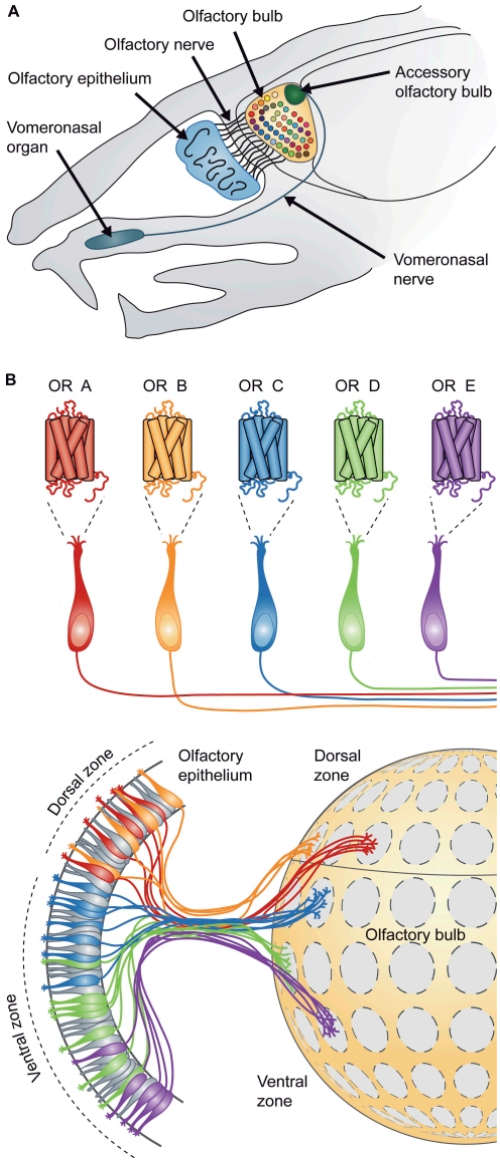


Figure 1. Schematic representation of adult rodent olfactory system. *A.* Axons of OSNs from the olfactory epithelium innervate the olfactory bulb. *B.* Each OSN encodes one OR gene, and the axons of all of the OSNs for a specific OR gene converge into a glomerulus in the olfactory bulb. The OSNs for an OR gene are organized dorsoventrally along the olfactory epithelium and then accumulate in a glomerulus in the corresponding dorsal-ventral area of the olfactory bulb. Figure 1 reused from © 2010 DeMaria and Ngai. Journal of Cell Biology. 191:443-452. doi: 10.1083/jcb. 201008163

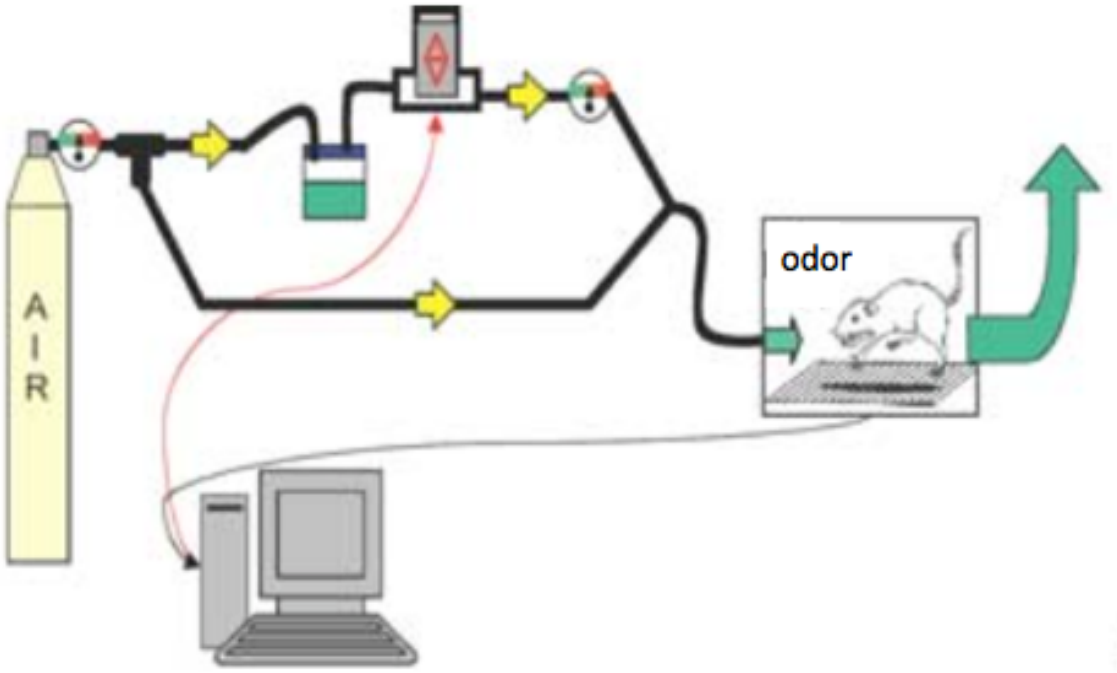


Figure 2. Experimental set-up of F0 fear conditioning and F1 testing. Both the SR-LAB Startle Response System and the Mouse HabiTest chambers allowed for compressed air to travel through a jar with the odor and subsequently a solenoid that permitted the odor to enter the chambers. Exhaust pipes or vacuums then forced the air and odor out of the chambers. Shocker floors were added to the chambers during fear conditioning. The MouseHabiTest chambers also contained a camera inside the chambers to record freezing behaviors, which was analyzed by the FreezeFrame software. Figure 2 modified with permission from © 2008 Jones et al. The Journal of Neuroscience. 28:13106-13111. doi: 10.1523/JNEUROSCI

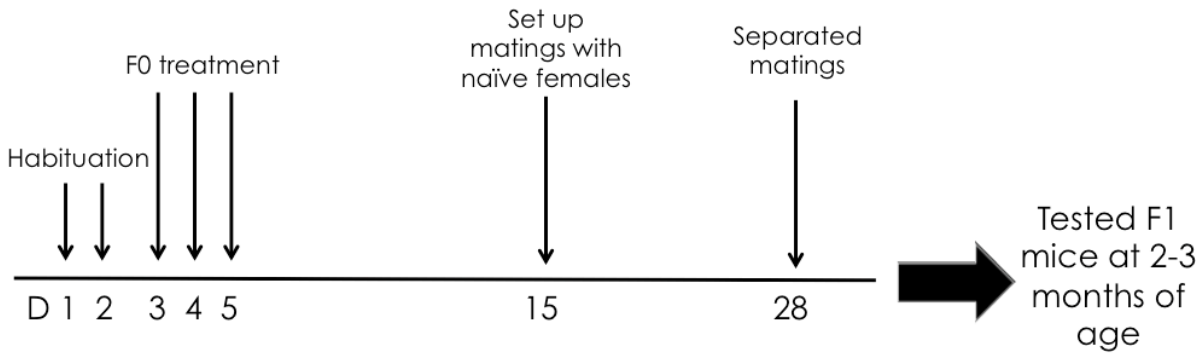


Figure 3. General experimental design. F0 mice were habituated to SR-LAB Startle Response System chambers for two consecutive days prior to treatment, which consisted of either 5 odor-footshock pairings over fifteen minutes once a day for three consecutive days (F0-Trained), 5 odor exposures over fifteen minutes once a day for three consecutive days (F0-Exposed), or kept in their home cages and subjected to occasional handling (F0-Home). Ten days later, all F0 mice were mated with naïve females, and thirteen days after mating set-ups, male F0 mice were removed from breeding cages. F1 mice were tested at two to three months of age.

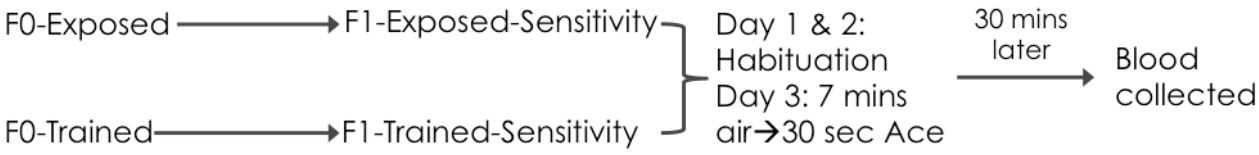


Figure 4. Experimental design to test for intergenerational olfactory sensitivity in the F1 generation. One set of F1 offspring of F0-Exposed and F0-Trained sires were habituated for two days to Mouse HabiTest chambers and then exposed to seven minutes of air followed by one 30-second presentation of acetophenone on day 3. Thirty minutes later, trunk blood was collected from all F1 animals to measure serum corticosterone levels.

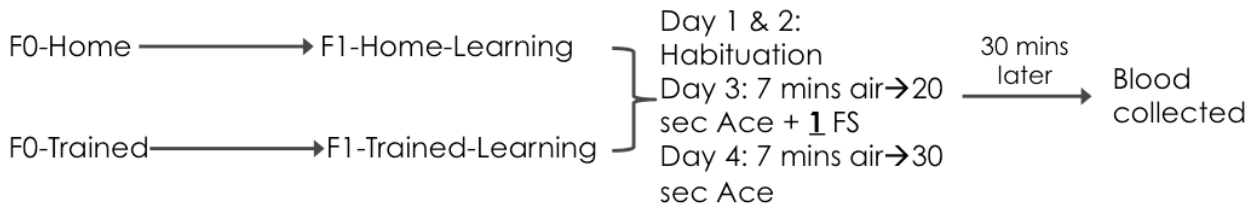
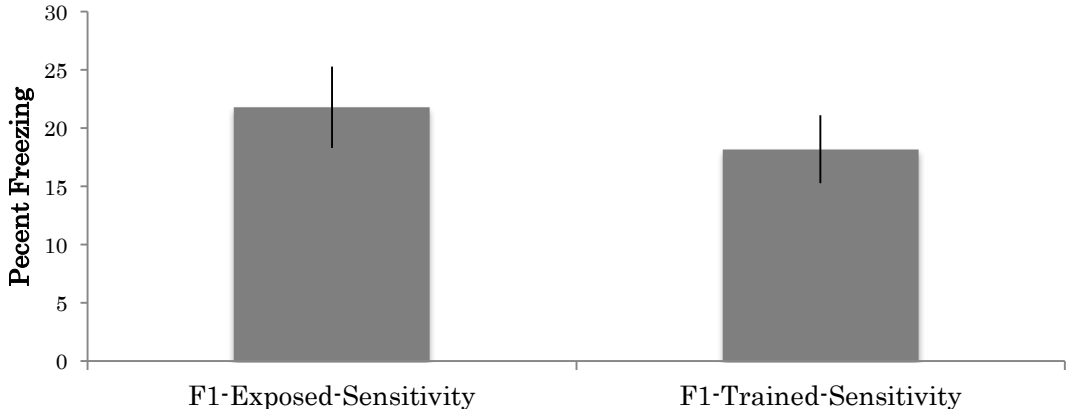


Figure 5. Experimental Design to test for altered threshold for learning in the F1 generation. Another set of F1 offspring of F0-Home and F0-Trained sires were habituated for two days to Mouse HabiTest chambers. On day 3, they were subjected one odor-footshock pairing, and twenty-four hours later, they were presented with the same odor. Blood was collected 30 minutes later from all F1 animals to measure serum corticosterone levels.

A



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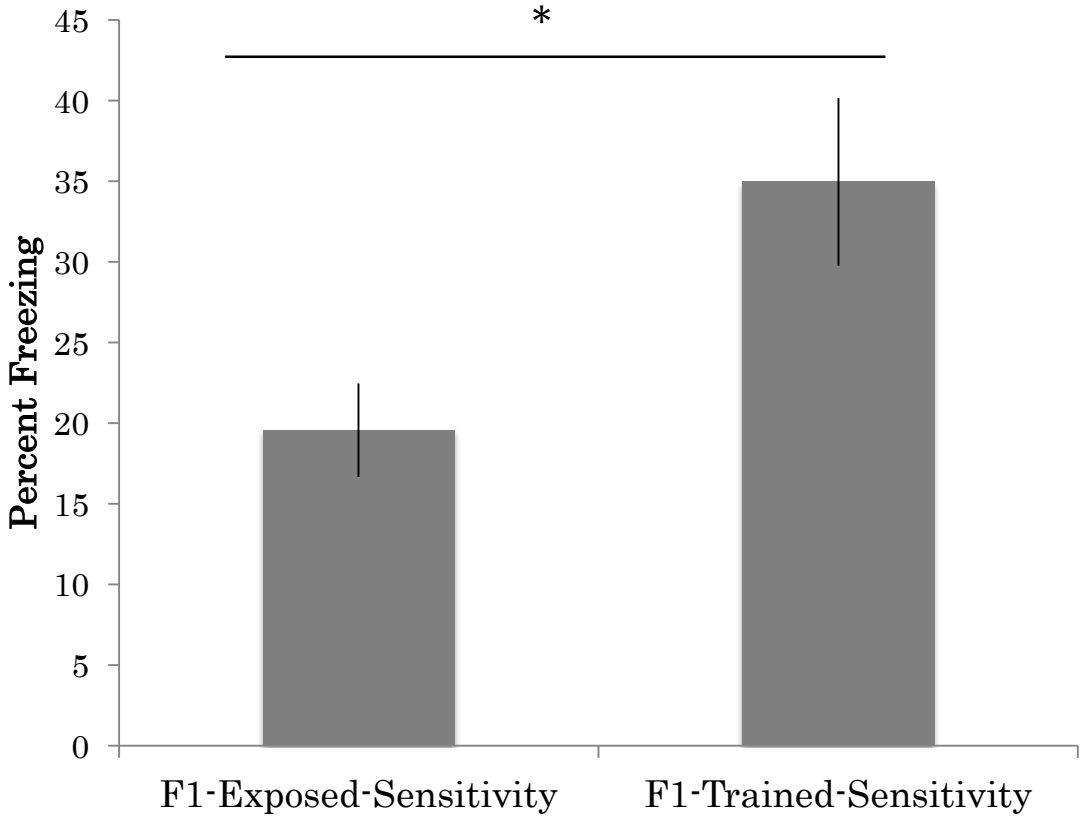
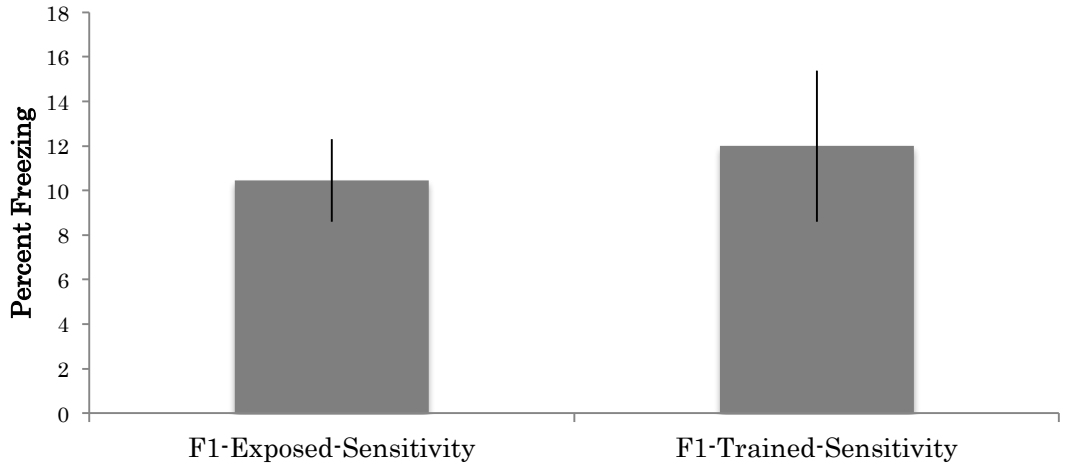


Figure 6. F1 male offspring of olfactory conditioned sires exhibited increased sensitivity and freezing towards the paternally conditioned odor. *A*. There was no significant difference in pre-odor freezing between F1-Exposed-Sensitivity males and F1-Trained-Sensitivity males. *B*. F1-Trained-Sensitivity **males** froze significantly more to an acetophenone presentation than F1-Exposed-Sensitivity males did. Columns and error bars represent mean ± S.E.M percent freezing (*$p < 0.05$).

A



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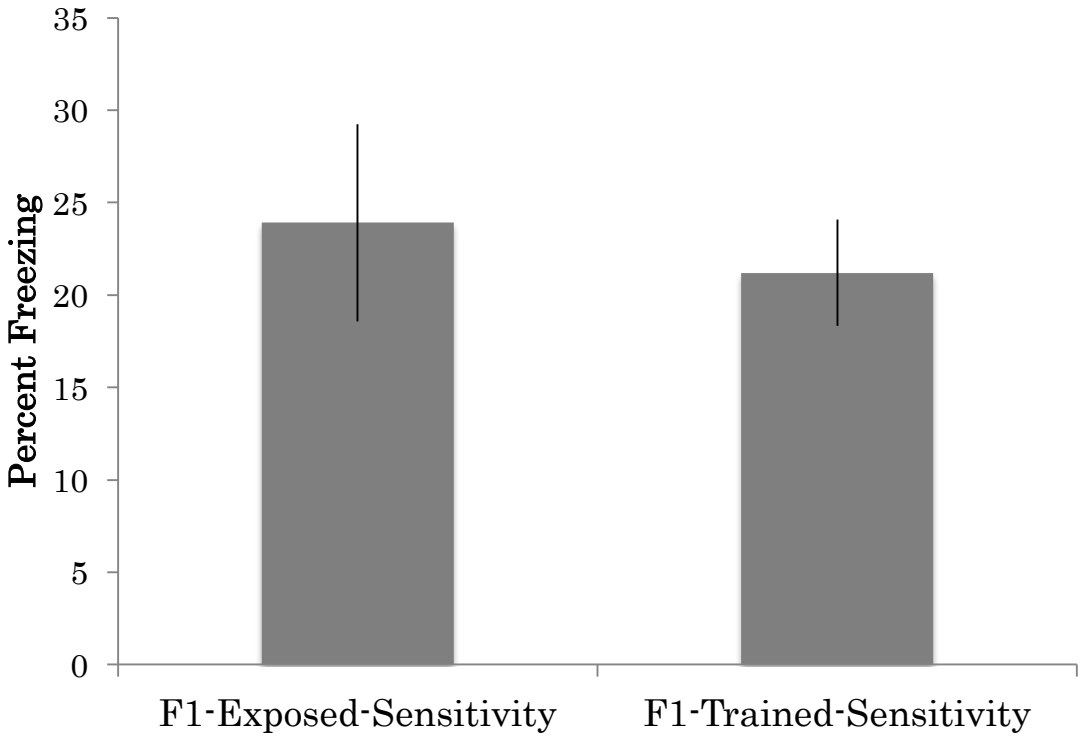


Figure 7. Female offspring of olfactory conditioned sires did not exhibit an enhanced sensitivity towards the paternally conditioned odor. *A*. There was no significant difference in pre-odor freezing between F1-Exposed-Sensitivity females and F1-Trained-Sensitivity females. *B*. There was no significant difference in percent freezing between F1-Exposed-Sensitivity **females** and F1-Trained-Sensitivity females during an acetophenone presentation. Columns and error bars represent mean ± S.E.M percent freezing.

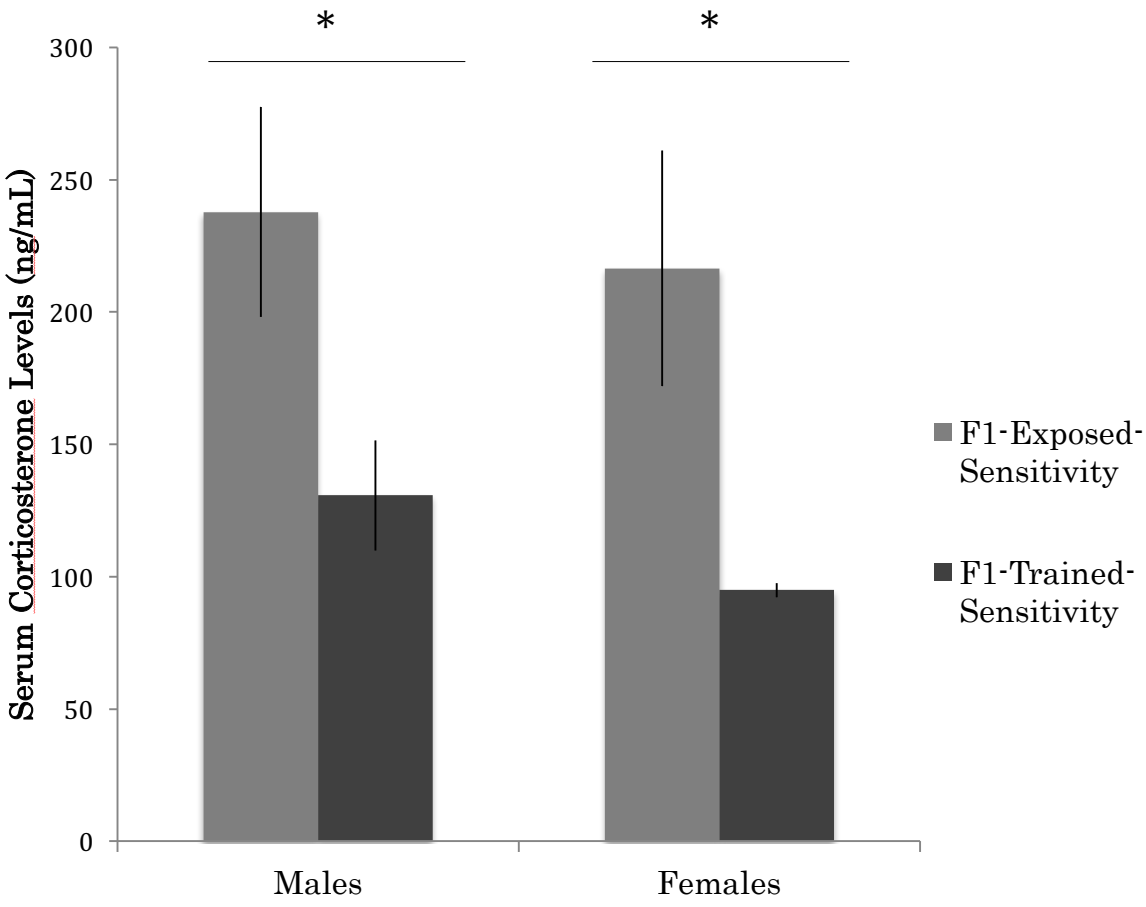
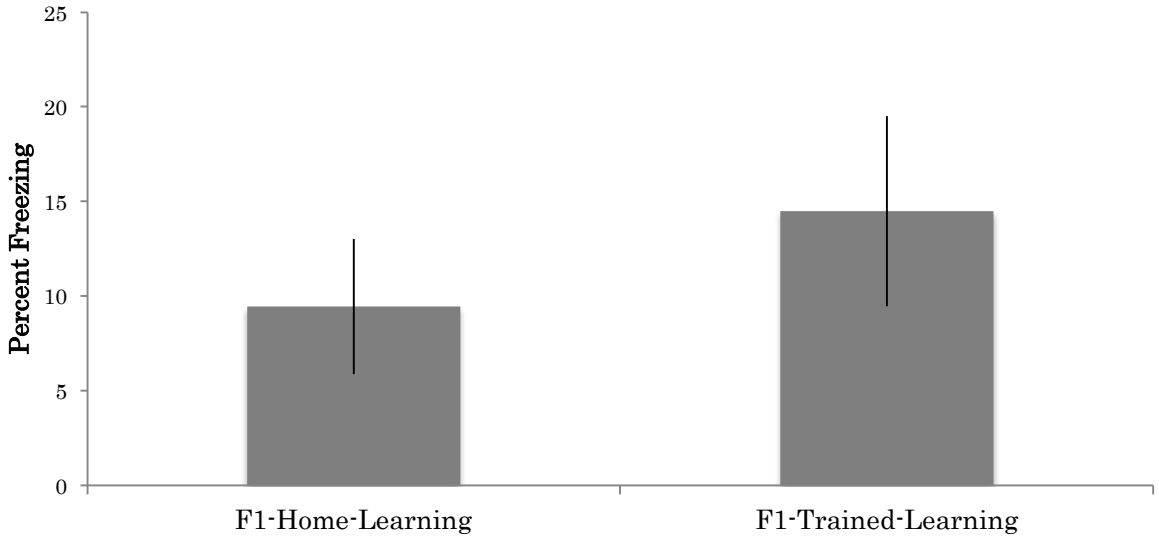


Figure 8. Both male and female offspring of olfactory fear conditioned mice exhibited a blunted corticosterone response after one exposure to the paternally conditioned odor. **Male and female** F1-Trained-Sensitivity mice exhibited significantly lower serum corticosterone levels than male and female F1-Exposed-Sensitivity mice, respectively, in response to an acetophenone presentation. Columns and error bars represent mean ± S.E.M serum corticosterone levels (*$p < 0.05$).

A



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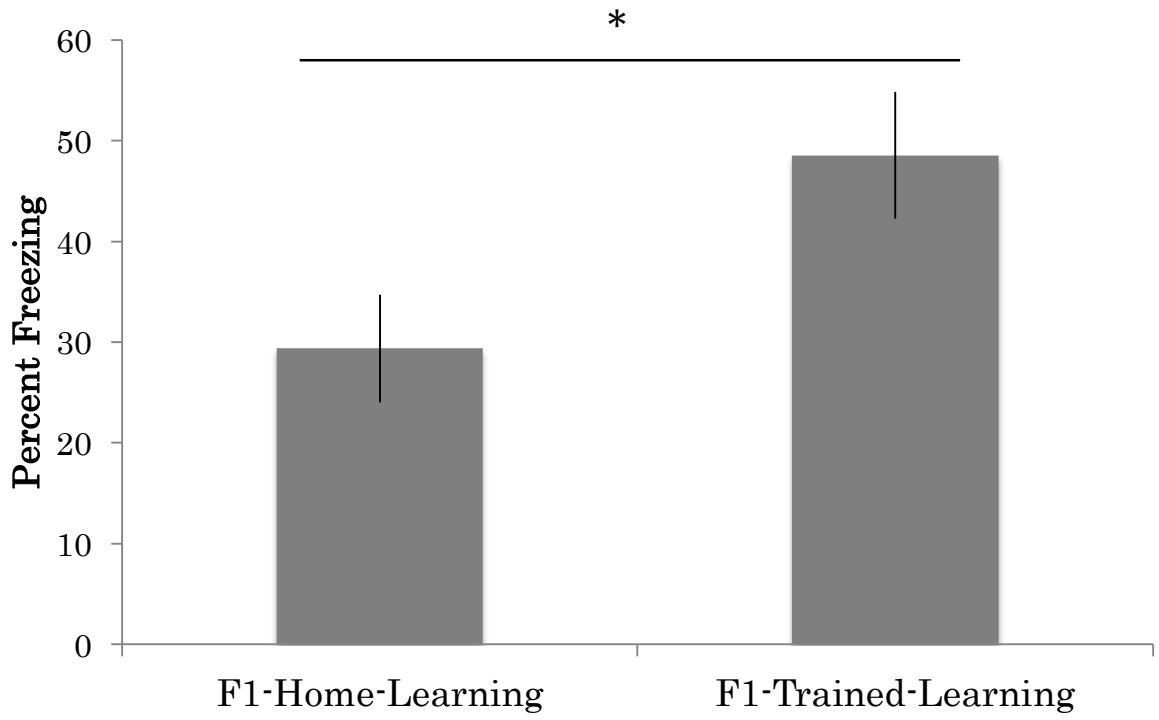
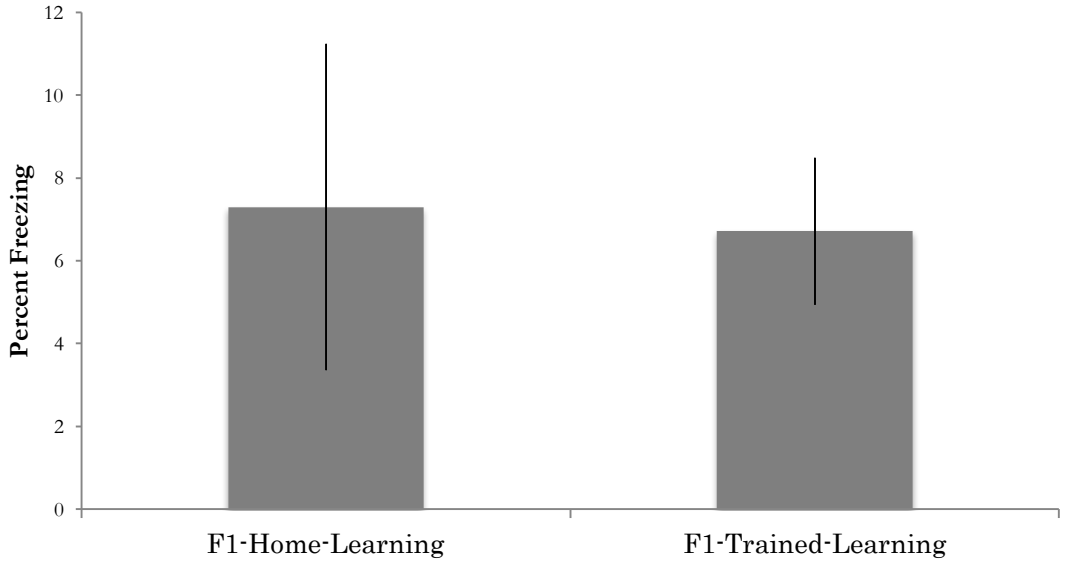


Figure 9. F1 male offspring have a reduced threshold for learning to paternally conditioned odor. *A.* There was no significant difference in pre-odor freezing between F1-Home-Learning males and F1-Trained-Learning males. *B.* F1-Trained-Learning **males** froze significantly more to acetophenone one day after being subjected to a weak conditioning paradigm with acetophenone. Columns and error bars represent mean ± S.E.M percent freezing (*$p < 0.05$).

A



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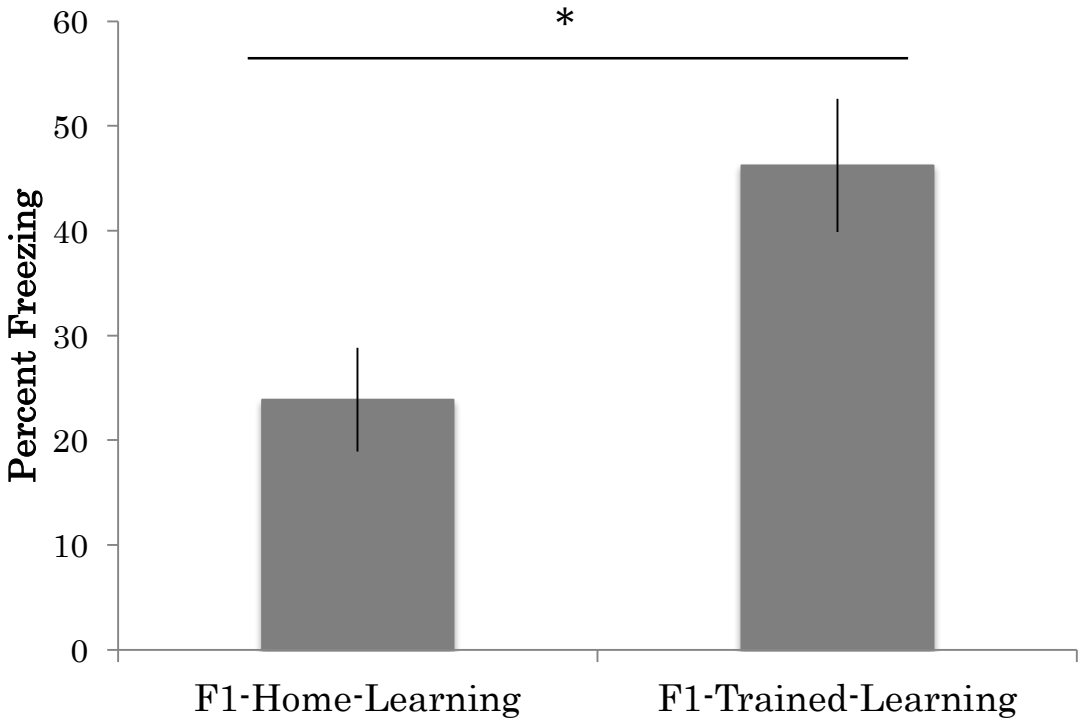

Figure 10. F1 female offspring have a reduced threshold for learning to paternally conditioned odor. *A.* There was no significant difference in pre-odor freezing between F1-Home-Learning females and F1-Trained-Learning females. *B.* F1-Trained-Learning **females** froze significantly more to acetophenone one day after being subjected to a weak conditioning paradigm with acetophenone. Columns and error bars represent mean ± S.E.M percent freezing (*$p < 0.05$).

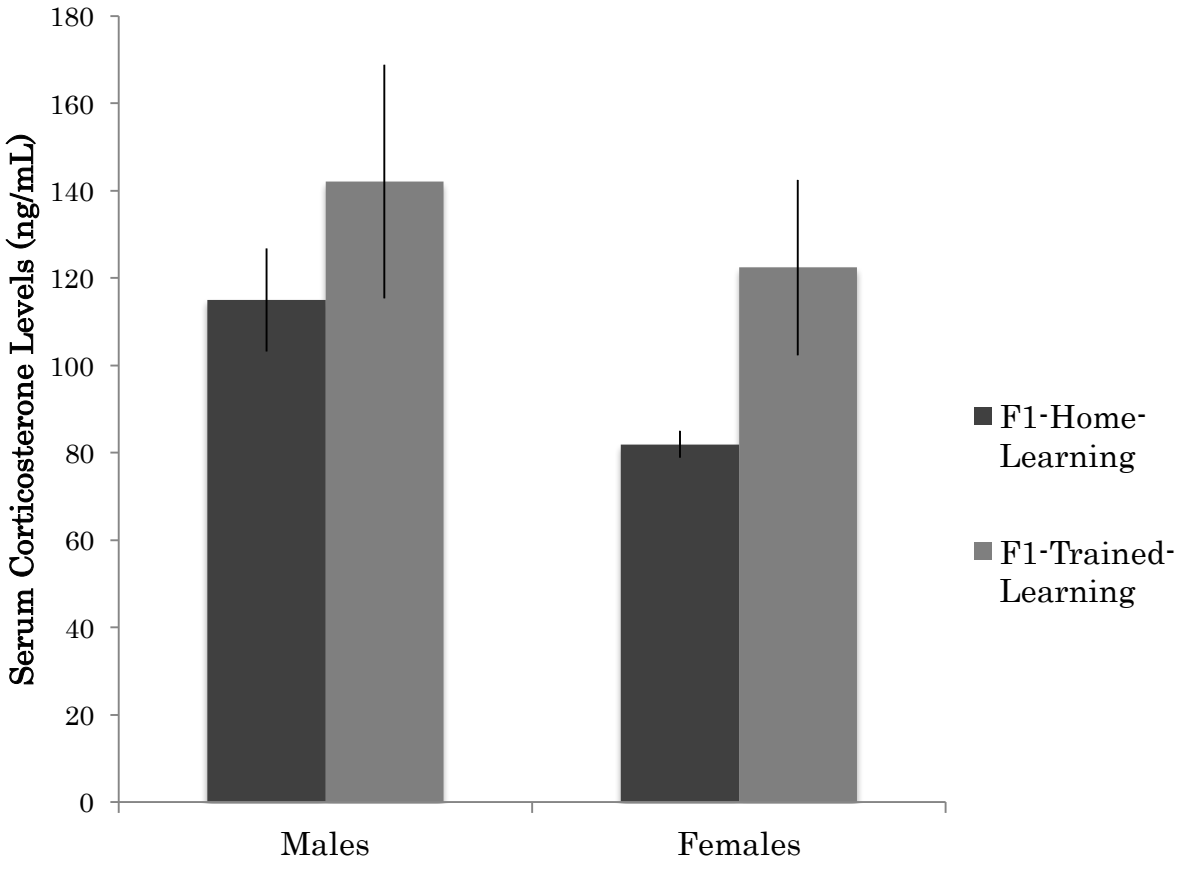

Figure 11. F1 male and female offspring of olfactory conditioned males do not mount an altered stress response due to a presentation of the paternally conditioned odor one day after being weakly conditioned it. There was no significant difference in serum corticosterone levels between **male** and **female** F1-Home-Learning and F1-Trained-Learning mice after an acetophenone presentation one day after being subjected to a weak conditioning paradigm to acetophenone.