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Intergenerational effects of paternal predator cue exposure on behavior, stress reactivity, and neural gene expression

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ABSTRACT

Predation threat impacts prey behavior, physiology, and fitness. Stress-mediated alterations to the paternal epigenome can be transmitted to offspring via the germline, conferring a potential advantage to offspring in predator-rich environments. While intergenerational epigenetic transmission of paternal experience has been demonstrated in mammals, how paternal predator exposure might alter offspring phenotypes across development is unstudied. We exposed male mice to a predator odor (2,4,5-trimethylthiazoline, TMT) or a neutral odor (banana extract) prior to mating and measured offspring behavioral phenotypes throughout development, together with adult stress reactivity and candidate gene expression in the prefrontal cortex, hippocampus, amygdala, and hypothalamus. We predicted that offspring of TMT-exposed males would be less active, would display elevated anxiety-like behaviors, and would have a more efficient stress response relative to controls, phenotypes that should enhance predator avoidance in a high predation risk environment. Unexpectedly, we found that offspring of TMT-exposed males are more active, exhibit less anxiety-like behavior, and have decreased baseline plasma corticosterone relative to controls. Effects of paternal treatment on neural gene expression were limited to the prefrontal cortex, with increased mineralocorticoid receptor expression and a trend towards increased *Bdnf* expression in offspring of TMT-exposed males. These results suggest that fathers exposed to predation threat produce offspring that are buffered against non-acute stressors and, potentially, better adapted to a predator-dense environment because they avoid trade-offs between predator avoidance and foraging and reproduction. This study provides evidence that ecologically relevant paternal experience can be transmitted through the germline, and can impact offspring phenotypes throughout development.

1. Introduction

Cues received during early development can shape adult phenotypes in predictable ways that range from constitutive effects on morphology (Brakefield et al., 1996; Agrawal et al., 1999; Beaty et al., 2016) to context-dependent neuroendocrine and behavioral responses (Liu et al., 1997; Solomon-Lane and Hofmann, 2019; Lehto and Tinghitella, 2019). When cues are accurate predictors of adult environments an appropriate developmental response should improve survival probability in that environment (Schmidt, 2011; Nederhof and Schmidt, 2012). Consistent with this prediction, *Daphnia* embryos exposed to chemical cues from a predatory water flea develop body armor that protects against this predator (Agrawal et al., 1999) and the offspring of gravid crickets exposed to a spider predator exhibit enhanced levels of anti-predator behavior and have higher survival relative to controls (Storm and Lima, 2010). In mammals, prenatal exposure to chronically elevated levels of corticosteroids, the end products of hypothalamus-pituitary-adrenal (HPA) axis activation in response to a stressor, has particularly strong effects on response to stressors in later life. In rodent models, offspring of mothers that were chronically stressed while pregnant are typically more anxious, more cautious, and more stress reactive than offspring of unstressed mothers (van Bodegom et al., 2017). However, whether these organizing effects of corticosteroids on HPA axis development are adaptive in a high stress environment is an open question. While there is evidence that early life stress can protect against the effects of chronic stress in adulthood, high anxiety and stress reactivity can also increase vulnerability to stress pathology, regardless of the level of stress in the adult environment (Meaney et al., 2007; Gluckman et al., 2008; Chen and Baram, 2016).

Whereas the stress experienced by pregnant females and offspring is

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simultaneous, stress-mediated alterations to the paternal epigenome can be transmitted to offspring via the germline (Rodgers et al., 2013; Dias and Ressler, 2014). Thus, models of paternal stress in which males experience stress before mating and never interact with their offspring allow separation of offspring response to paternal cues from the strong directional effects of maternal corticosteroids on brain development. The proposed mechanisms by which individual experience is transduced to heritable changes in epigenetic modifiers of gene expression (e.g. DNA methylation, sperm microRNAs) are diverse (reviewed in Bohacek and Mansuy, 2015), and the effects of paternal stress on offspring phenotypes can be highly specific. For example, offspring of male mice presented with a neutral odor paired with an electrical shock displayed a fear response to the same odor and had altered DNA methylation on the olfactory receptor that binds the odor's main ligand (Dias and Ressler, 2014). Moreover, paternal exposure to less acute but ecologically and physiologically relevant stressors, such as changes in food availability (Mashoodh et al., 2018), diet composition (Carone et al., 2010; Weyrich et al., 2018), heat exposure (Weyrich et al., 2016), and predation threat (Korgan et al., 2016), also produces altered epigenetic and gene expression phenotypes in offspring (reviewed in Champagne, 2019). Collectively, these studies indicate that the paternal germline can transmit epigenetic information between generations, that this information can be influenced by environmental conditions, and that these inherited epigenetic changes could impact offspring fitness in nature.

An increase in predation threat, whether real or perceived, has profound effects on prey behavior, physiology, and fitness. Experimental manipulation of perceived predation threat reduces prey species' foraging activity (Peacor and Werner, 2000), reproductive output (Nelson et al., 2004; Creel et al., 2007; Sheriff et al., 2009; Travers et al., 2010; Zanette et al., 2011), and parental investment (Dudek et al., 2018), and can increase prey mortality in the absence of direct contact with the predator (McCauley et al., 2011; MacLeod et al., 2017). In natural systems, parental perception of high predation threat can reduce offspring survival (Sheriff et al., 2009; Zanette et al., 2011). Conversely, there is also evidence for a protective effect of the predation threat experienced by parents on offspring stress response (Fisher et al., 2014; Kärkkäinen et al., 2019), and for parental programming of offspring behaviors (Giesing et al., 2011; Morales et al., 2018) and stress reactivity (Brachetta et al., 2018) that should promote survival in a high predation environment.

In laboratory rodents, the offspring of females exposed to chronic predation threat during gestation are generally cautious and stress-reactive and exhibit increased anxiety-related behaviors (St-Cyr and McGowan, 2015; St-Cyr et al., 2017, 2018), all phenotypes that could be adaptive when predators are common. However, these phenotypes are much like those produced by maternal exposure to other types of stressors, presumably due to the shared programming effects of exposure to elevated corticosteroids in utero. This raises the question of whether corticosteroid-independent transmission of paternal predation experience produces similarly stress-reactive offspring. To our knowledge, only two prior studies have tested for effects of paternal predation threat on offspring. Whereas Korgan et al. (2016) found evidence for increased boldness in the juvenile offspring of male rats exposed to cat odor, Azizi et al. (2019) found increased anxiety in the juvenile offspring of rats exposed to a live cat, regardless of whether mothers, fathers, or both were cat-exposed prior to mating. These discordant results suggest that differences in the nature of paternal predator exposure (e.g. unimodal vs. multimodal predator cues) can lead to opposing effects on offspring phenotypes, motivating further work on this topic. Moreover, no study to date has evaluated whether the direction and strength of these paternal effects are stable across development.

Here, we investigate the effects of paternal exposure to predator odor on offspring activity and anxiety-like phenotypes throughout development, and on stress reactivity and neural gene expression in adulthood. We chronically exposed adult male mice to an ecologically-relevant dose of 2,4,5-trimethylthiazoline (TMT), a component of fox feces that is aversive to rodents and widely used in predation stress paradigms (Buron et al., 2007; Hacquemand et al., 2013; Janitzky et al., 2015; St-Cyr and McGowan, 2015; St-Cyr et al., 2017; Green et al., 2018). We hypothesized that offspring of males exposed to predator cues prior to mating would exhibit 1) behaviors that should promote survival in a high predation environment relative to offspring of control males and 2) hormonal phenotypes consistent with a more efficient stress response relative to offspring of control males. Specifically, we predicted that offspring of males exposed to predator cues would exhibit less exploratory behavior and increased anxiety-like behaviors relative to offspring of control males, would exhibit greater avoidance when presented with the same predator cue, and would have higher corticosterone levels under acute stress with faster return to basal levels.

We also measured mRNA expression for five stress-associated genes (*Nr3c1, Nr3c2, Drd1, Drd2, Bdnf*) in brain regions that modulate stress reactivity (prefrontal cortex, hippocampus, amygdala, hypothalamus). We predicted that offspring of males exposed to predator odor would have higher glucocorticoid receptor (*Nr3c1*) expression in the hippocampus and prefrontal cortex, promoting more efficient termination of the stress response and protecting against the deleterious effects of chronic stress. We did not make directional predictions for the other four genes but expected to detect effects of paternal stress based on the following reasoning. Corticosteroid binding to the mineralocorticoid receptor (*Nr3c2*) facilitates organization of cognitive processes and assessment of the environment after the stress response is initiated (Schwabe et al., 2013), and interactions between glucocorticoids and Nr3c2 are critical determinants of the threshold at which an acute stress response is launched (de Kloet et al., 2019). We included dopamine receptors *Drd1* and *Drd2* because, in addition to its function in reward circuitry, dopamine encodes aversion to stressful stimuli, especially when the fear response is initiated (Pignatelli and Bonci, 2015). Finally, brain derived neurotrophic factor (*Bdnf*) promotes neuronal growth and differentiation in the central nervous system (Osório et al., 2017). Both acute stress and stress-related disorders alter *Bdnf* expression in humans (Denhardt, 2018; Osório et al., 2017), and chronic stress paradigms in rodents cause decreased *Bdnf* expression (Shi et al., 2010).

2. Methods

2.1. Mouse husbandry

Mice were housed in polycarbonate cages with Sanichip® bedding and ad libitum access to food (LabDiet® 5001 Rodent Diet) and water. Adult males (paternal generation) were singly-housed except when paired with a female. Offspring were weaned into same sex groups of up to 4 individuals/cage. The colony was maintained on a 12-hour light:dark cycle with lights on at 0930. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at Oklahoma State University.

2.2. Paternal treatment

Sexually naïve, adult male C57BL/6J mice (age range: 62–150 days, mean ± SD: 103.2 ± 2.9 days) were exposed to either 10% TMT (BioSRQ, SKU 1G-TMT-90) in propylene glycol (experimental; $n = 15$) or 1% banana extract in propylene glycol (control; $n = 13$) 5 min daily for 8 consecutive days. Rodents do not habituate to TMT (Green et al., 2018) and chronic exposure produces lasting anxiety-like and endocrine responses (Figueiredo et al., 2003; Apfelbach et al., 2005). We chose a 10% dilution because prior work demonstrates that 10% TMT induces levels of avoidance and anxiety-like behaviors that are statistically indistinguishable from the behavioral effects of fresh fox feces

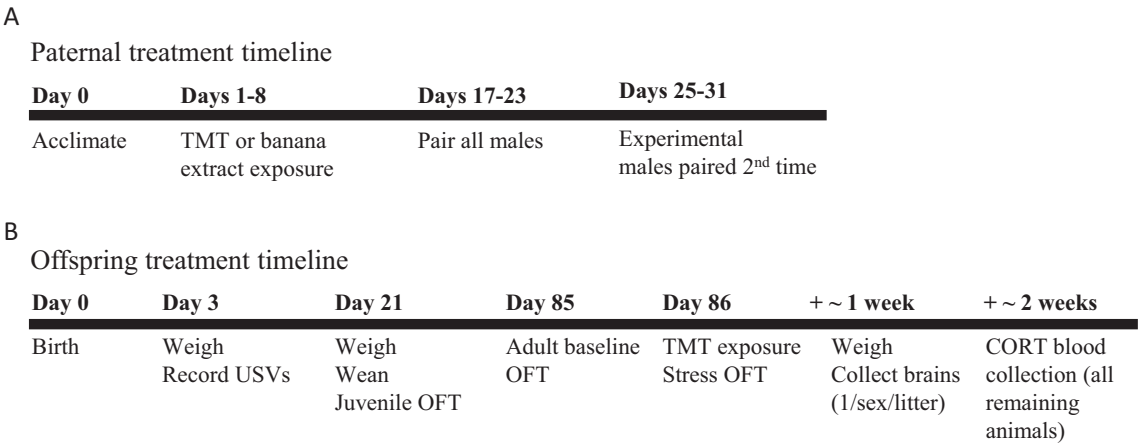

Fig. 1. Experimental timelines for A) paternal and B) offspring treatments. USVs, ultrasonic vocalizations; OFT, open field test. In B, +~1 week and +~ 2 weeks reference time between last behavioral assay and brain and blood collection, respectively.

(Buron et al., 2007). Mice were housed in a separate room (to avoid exposing the main mouse colony to TMT) for the duration of the exposure period, with 24 hour acclimation prior to the first exposure. Treatment (50 µl/day TMT or banana extract) was administered on filter paper affixed to a glass dish with double-sided tape, placed in the home cage for 5 min within the first 3 h of the light cycle (0930–1230 h). Daily exposures were video recorded and scored blind to treatment. To confirm that TMT elicited a behavioral response we scored time spent on the cage side with the odor on exposure day 1, and tested for an effect of odor identity with one-way analysis of variance (ANOVA). Effect size was calculated with eta squared.

Each TMT-exposed male was paired twice with sexually naïve females (age range: 60–234 days, mean ± SD: 115.6 ± 5.8 days), at 17 and 25 days after first exposure to the predator cue (Fig. 1A). Spermatozoa that were mature at 17 and 25 days post-exposure were at the postmeiotic round spermatid and premeiotic spermatogonial stages, respectively, at first exposure (Oakberg, 1956; Fallahi et al., 2010). This design allowed us to test for an effect of spermatogenic stage during paternal TMT exposure on offspring phenotypes. Control males were paired once at 17 days after first exposure (Fig. 1A). All pairs were split after 6 days to minimize potential effects of male behavior on mothers. Females were transferred to a fresh cage with a cotton nestlet and paper hut and left undisturbed, except for routine handling associated with cage changes. Cages with females that were visibly pregnant (10–12 days following splitting pairs) were checked daily for pups by visual inspection. In the offspring of TMT-exposed males, we evaluated the effect of paternal spermatogenic stage on behavior, gene expression, and CORT using linear mixed models, with timing of pairing as a fixed effect and litter ID as a random effect (Supplementary Table S1).

2.3. Offspring behavioral assays

Offspring were used in a series of behavioral assays, from post-natal day (PND) 3 through adulthood, before sacrifice as adults for either tissue or blood collection (Fig. 1B). On PND 3, we weighed offspring and recorded ultrasonic vocalizations (USVs) for 3 min. Neonatal USVs promote maternal retrieval when neonates are displaced from the nest (Mogi et al., 2017), and are used as a measure of anxiety in infant rodents (Winslow, 2009). Pups were removed individually from the nest and placed in a cage with clean bedding inside the recording chamber, a 52 × 36 × 30 cm anechoic foam-lined PVC box with a microphone (UltraSoundGate CM16/CMPA, Avisoft Bioacoustics) positioned ~15 cm above the floor of the box. Recording began immediately after placement and vocalizations were sampled at 192 kHz, 16 bits using Avisoft (version 4.2.24) software and hardware (UltraSoundGate 116hb). The number of vocalizations (distinct notes) per minute over a three minute period was scored manually in Raven Pro (version 1.4).

On PND 21, offspring were weighed and weaned into same-sex sibling groups, and five-minute open field tests (OFTs) were conducted during the lights-on part of the light cycle (85 lx) between 1000 and 1400 h. We chose this test because it measures behaviors that are salient to predator avoidance in mice: exploration of a novel environment (activity) and avoidance of open spaces (time in center) (Gould et al., 2009). In five-minute OFTs, both activity and time in center are potentially indicative of the relative level of anxiety (Gould et al., 2009) or fear (Ennaceur, 2014) elicited by exposure to an unfamiliar and unprotected environment. Lower activity and less time in center are suggestive of higher relative levels of anxiety or fear in this context (Gould et al., 2009).

The open field arena consisted of a 16-square grid enclosed by a clear Plexiglas box with no lid (60.96 cm × 60.96 cm × 60.96 cm). To ensure that all animals started in the same location, mice were placed in a vertically oriented opaque PVC tube in the center of the arena, and each five-minute trial began after the mouse left the center of the grid following removal of the tube. Trials were video recorded and activity (total number of gridlines crossed) and time spent in the four central squares of the grid were scored by an observer blind to treatment.

At approximately PND 85 (mean ± SD: 84.6 ± 0.85), all offspring were exposed to 50 µl 10% TMT for 5 min to measure fear response to the same predator cue experienced by experimental fathers. We did not include a control odor (e.g. banana extract) because our goal was to test for an effect of paternal predator odor exposure on offspring response to TMT. As for fathers, TMT was presented on filter paper affixed to a glass dish and mice were exposed in a separate room to avoid colony exposure to TMT. We randomized which side of the cage the filter paper was on in each trial to eliminate side bias and used a clean cage with fresh bedding for each trial. Trials were video recorded and scored blind to treatment. Response was measured by 1) calculating the amount of time spent on the side of the cage containing TMT relative to the side without and 2) scoring the following behaviors: sniffing the TMT filter paper, touching the TMT filter paper, rearing, and digging. OFTs were conducted the day before and immediately after TMT exposure to measure adult baseline and post-predator cue exposure (TMT stress) behaviors, respectively.

The total number of USVs was analyzed with a generalized linear mixed model including paternal treatment as a fixed effect and litter ID as a random effect using a Poisson distribution. Remaining USV data (first minute, second minute, latency to first call) were analyzed with linear mixed models, with paternal treatment as a fixed effect. Because paternal ID and litter ID were highly correlated, we opted to use only litter ID as a random effect in all models. Open field behaviors were analyzed with generalized linear mixed models incorporating paternal

treatment and sex as fixed effects, and litter ID as a random effect using a Poisson distribution. TMT behavioral data were analyzed with a linear mixed model with Satterwaithe approximation including paternal treatment and sex as fixed effects and litter ID as a random effect (Supplementary Table S2). We ran a linear mixed model on the ratio of baseline:TMT stress behaviors to determine the effect of paternal treatment on the change in open field behavior. Models were selected using the Akaike information criterion adjusted for small sample sizes (AICc). Effect sizes for GLMMs and LMMs were calculated using R^2c, which accounts for fixed and random effects (Nakagawa and Schielzeth, 2013). All analyses were conducted in R Version 3.5.2 "Eggshell Igloo".

2.4. Neural tissue collection and qPCR

One week following the post-TMT open field test (mean age ± SD: 86.9 ± 2.5 days), one individual per sex per litter was sacrificed by cervical dislocation. Brains were extracted into RNAlater, stabilized at 4 °C for 24–48 h, and stored at −20 °C until dissection. Target brain regions (amygdala, hypothalamus, hippocampus, prefrontal cortex) were dissected into RNAlater with a scalpel under a dissecting microscope (Zapala et al., 2005; Chiu et al., 2007), using the mouse brain atlas (Paxinos and Franklin, 2013) as a guide; bregma coordinates are provided in Supplementary Table S3.

RNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was converted to cDNA with Bio-Rad iScript Reverse Transcriptase Supermix for RT-qPCR (cat. no: 1708841) with target RNA input of 0.6 μg (prefrontal cortex), 0.5 μg (amygdala and hippocampus), or 0.3 μg (hypothalamus). Primers for qPCR (Supplementary Table S4) were designed in Primer-BLAST (Ye et al., 2012) using sequences and intron-exon structure from Ensembl Mouse (GRCm38.p6). When feasible, primers spanned an intron-exon boundary. Experiments were performed on a CFX Connect Real-Time System (Bio-Rad). Each 10 μl reaction contained 4 μl cDNA (diluted 1:10), 5 μl SsoAdvanced™ Universal® SYBR Green Supermix (Bio-Rad; cat. no: 1725270), and 0.3 μM of each primer. Plates were balanced for sex and paternal treatment and included three technical replicates per sample for experimental genes and the internal control (beta-actin). Relative mRNA expression was calculated using the comparative C_T method (Schmittgen and Livak, 2008), with threshold crossing (C_T) values normalized relative to beta-actin. We tested for an effect of paternal treatment and sex on gene expression with ANOVA. To account for tests on each gene across four brain regions, alpha was set to 0.0125 using a Bonferroni correction. Effect sizes were calculated using eta squared.

2.5. Blood collection and corticosterone assay

Approximately two weeks after all behavioral tests had been completed (mean age ± SD: 103.2 ± 1.6 days) blood for baseline CORT measurements was collected into heparinized tubes from the submandibular vein for all remaining offspring. Two to five days later, the same individuals were stressed by 30 minute restraint inside a small plastic tube (Kaytee Critter Trails Fun-nels, 6.35 cm diameter, 8.85 cm long) placed in their home cage. Immediately after removal from the tube, a second blood sample (stressed sample) was collected by submandibular bleeding. One hour later, mice were sacrificed by cervical dislocation and a third sample was collected by cardiac puncture (recovery sample). Blood samples were spun down (5 min at 8500 rpm in an Eppendorf 5424 benchtop centrifuge) and plasma was stored at −80 °C. Baseline samples were collected between 1200 and 1300 and the post-restraint stress sample for a given individual was collected at the same time of day as the baseline. All samples were collected within 2 min of opening the cage at each time point.

CORT levels were assayed with an ELISA (Enzo Life Sciences; cat. no: ADI-900-097) optimized for mouse plasma (1,40 plasma dilution, 1% steroid displacement buffer). Plates were read at 405 nm on a Biotek EL808 plate reader. Standards were run in triplicate and samples were run in duplicate. Plasma was pooled from same-sex littermates to provide sufficient volume for the assay. Sample sizes for litters sired by TMT-exposed males were $n = 15$ baseline, $n = 13$ stressed, and $n = 14$ recovery. Sample sizes for control litters were $n = 9$ baseline, $n = 10$ stressed, and $n = 11$ recovery. The inter-plate coefficient of variation (CV) was 5.00% and average intra-plate CV was 7.74%. To determine whether offspring of predator cue-exposed and control males differed in baseline glucocorticoid levels, an effect of paternal treatment on baseline CORT was tested with ANOVA, and effect size was tested with eta squared. The effect of paternal treatment on CORT levels at all three time points was tested with a linearized mixed model with a Satterwaithe approximation and a Poisson distribution, incorporating time point and paternal treatment as fixed effects with litter as a random effect (Supplementary Table S5). We tested for an effect of sex using a linearized mixed model with Satterwaithe approximation incorporating sex, time point, and paternal treatment as fixed effects with litter as random effect (Supplementary Table S5). LMM effect size was tested with R^2c.

3. Results

3.1. Paternal response to TMT

TMT exposure elicited strong avoidance behavior whereas exposure to the control odor (banana extract) did not. On exposure day 1, males in the TMT treatment spent, on average, 19% of the 5 minute exposure period on the side of the cage with TMT (mean ± SD: 57 s ± 22.3) whereas males in the control treatment spent an average of 47% of the exposure period on the side of the cage with banana extract (mean ± SD: 141 s ± 73.0). This difference was highly significant (ANOVA, $F_{1, 18} = 11.40$, $p = 0.0034$, $\eta^2 = 0.27$; Fig. S1).

3.2. Effect of time of paternal pairing on offspring phenotypes

There was no effect of when TMT-exposed males were paired (17 vs. 25 days after first TMT exposure) on offspring USVs, open field behavior, or behavior during TMT exposure (first pairing: $n = 15$, second pairing: $n = 6$, LMM, all $p > 0.15$), nor was there an effect on offspring mean CORT concentration at baseline, stressed, or during recovery (first pairing: $n = 9$, second pairing: $n = 6$, LMM, all $p > 0.3$). Of the litters sired by TMT-exposed males that were included in the analysis of neural gene expression, only one was the product of a second pairing. Therefore, litters conceived at the two post-TMT exposure time points were analyzed together.

3.3. Offspring body mass

Offspring of males exposed to predator odor did not differ in mass from control offspring as neonates (experimental $n = 19$ litters$^{117\ \text{individuals}}$, control $n = 10$ litters$^{59\ \text{individuals}}$; Fig. S2A), juveniles (experimental $n = 16$ litters$^{88\ \text{individuals}}$, control $n = 9$ litters$^{50\ \text{individuals}}$; Fig. S2B) or adults (experimental $n = 20$ litters$^{39\ \text{individuals}}$, control $n = 12$ litters$^{19\ \text{individuals}}$; Fig. S2C) (all $p > 0.4$). Male offspring were heavier than female offspring as juveniles (males $n = 50$, females $n = 88$; LMM, estimate ± SE: 0.46 ± 0.16, $t = 2.93$, $p = 0.0041$), and as adults (males $n = 26$, females $n = 32$; LMM, estimate ± SE: 3.88 ± 0.38, $t = 10.34$, $p < 0.001$), regardless of paternal treatment.

3.4. Ultrasonic vocalizations

Offspring of TMT-exposed males (19 litters$^{120\ \text{individuals}}$) and control males (11 litters$^{60\ \text{individuals}}$) did not differ in the total number of vocalizations produced during a 3-minute trial (GLMM, $p = 0.771$; Fig. S3). There was no effect of paternal treatment on the number of vocalizations produced in the first minute, or the second minute, or on

latency to the first call (LMM, all $p > 0.4$).

3.5. TMT assay

There was no effect of paternal treatment on adult offspring behaviors in the TMT assay (experimental, $n = 18$ litters$^{89\ individuals}$; control, $n = 11$ litters$^{59\ individuals}$; LMM $p > 0.3$; Fig. S4). There was a significant effect of sex on the amount of time spent on the side of the cage with TMT: male offspring spent less time on the TMT side than females regardless of paternal treatment (LMM, estimate ± SE: -11.93 ± 5.37, $t = -2.22$, $p = 0.026$, $R^2c = 0.036$). Similarly, female offspring tended to touch the TMT filter paper more often than males (LMM estimate ± SE: -1.703 ± 0.954, $t = -1.785$, $p = 0.07$, $R^2c = 0.024$). There were no effects of paternal treatment or sex on the number of times individuals sniffed TMT, reared, or dug during the 5 minute trial (LMM, all $p > 0.3$).

3.6. Open field tests

In the juvenile open field test, there were significant effects of sex (GLMM, estimate ± SE: -0.11 ± 0.018, $z = -5.76$, $p < 0.001$, $R^2c = 0.53$) and paternal treatment (GLMM, estimate ± SE: -0.46 ± 0.12, $z = -3.82$, $p = 0.001$, $R^2c = 0.53$) on number of lines crossed (Fig. 2A). Offspring of males with predator odor experience (15 litters$^{77\ individuals}$) were more active than offspring of control males (10 litters$^{52\ individuals}$). Within groups, females (experimental: 15 litters$^{48\ individuals}$, control: 10 litters$^{35\ individuals}$) were more active than males. Time in the center of the open field was best explained by a model incorporating the interaction between paternal treatment and sex (Fig. 2B). Offspring of TMT-exposed males spent more time in the center relative to control offspring (GLMM, estimate ± SE: -0.49 ± 0.15, $z = -3.37$, $p < 0.001$, $R^2c = 0.039$). This effect was driven by male offspring of TMT-exposed males, who spent the most time in the center relative to all other groups (GLMM, estimate ± SE: $0.22 \pm 0.0.068$, $z = 2.25$ $p = 0.001$, $R^2c = 0.039$, Fig. 2B).

In the adult offspring baseline (pre-TMT exposure) open field test, we found significant effects of sex (GLMM, estimate ± SE: -0.091 ± 0.015, $z = -6.02$, $p < 0.001$, $R^2c = 0.26$) and paternal treatment (GLMM, estimate ± SE: 0.13 ± 0.062, $z = 2.03$, $p = 0.042$, $R^2c = 0.26$) on number of lines crossed (Fig. 3A). Within groups, females were more active than males and offspring of TMT-exposed males (18 litters$^{89\ individuals}$) were more active than control offspring (11 litters$^{60\ individuals}$, Fig. 3A). Offspring of TMT-exposed males tended to spend more time in the center relative to controls (GLMM, estimate ± SE: 0.16 ± 0.089, $z = 1.82$, $p = 0.069$, $R^2c = 0.20$, Fig. 3B.

Following predator cue exposure, open field activity was best explained by the model incorporating the effects of paternal treatment, sex, and treatment by sex (Fig. 3A). Offspring of TMT-exposed males crossed more lines relative to control offspring (GLMM, estimate ± SE: 0.17 ± 0.076, $z = 2.24$, $p = 0.025$, $R^2c = 0.26$). Overall, males crossed more lines than females (GLMM, estimate ± SE: 0.055 ± 0.027, $z = 2.024$, $p = 0.043$, $R^2c = 0.26$). However, the direction of sex-specific effects was treatment-dependent: daughters of control males crossed significantly fewer lines relative to other groups, whereas sons were not affected by treatment (GLMM, estimate ± SE: -0.12 ± 0.034, $z = -3.54$, $p < 0.001$, $R^2c = 0.26$). There were similar effects of paternal treatment, sex, and treatment by sex on offspring time in center following predator cue exposure (Fig. 3B). Collectively, offspring of TMT-exposed males spent more time in the center relative to offspring of control males (GLMM, estimate ± SE: 0.32 ± 0.12, $z = 2.7$, $p = 0.0067$, $R^2c = 0.21$), and female offspring spent more time in the center than males (GLMM, estimate ± SE: 0.16 ± 0.067, $z = 2.32$, $p = 0.02$, $R^2c = 0.21$). This sex-specific effect was driven by the daughters of TMT-exposed males: these females spent the most time in the center relative to all other groups whereas daughters of control males spent the least time in the center (GLMM, estimate ± SE: -0.18 ± 0.081, $z = -2.25$, $p = 0.024$, $R^2c = 0.21$, Fig. 3B).

Neither paternal treatment nor sex affected the change in behavior following exposure to the predator cue, measured as the ratio of line crosses or time spent in center before and after TMT exposure (LMM, $p > 0.2$; Fig. S5).

To evaluate the possibility that time spent in the center of the open field was a byproduct of activity level rather than a discrete behavior, we tested for correlations between the time each mouse spent in the center of the apparatus and the number of lines it crossed. We ran a total of 12 tests (2 sexes × 2 treatment groups × 3 OFTs), of which 11 were non-significant ($p > 0.15$, $R^2 < 0.1$; Figs. S6 and S7). Time in center and lines crossed were significantly positively correlated in adult control females, post-TMT exposure ($p = 0.02$, $R^2 = 0.155$; Fig. S7).

3.7. Corticosterone assay

Baseline plasma CORT was significantly lower in offspring of TMT-exposed males relative to offspring of control males (ANOVA, $F_{1, 23} = 5.32$, $p = 0.026$, $\eta^2 = 0.12$, Fig. 4A). However, there was no effect of paternal treatment across all three time points (LMM, estimate ± SE:

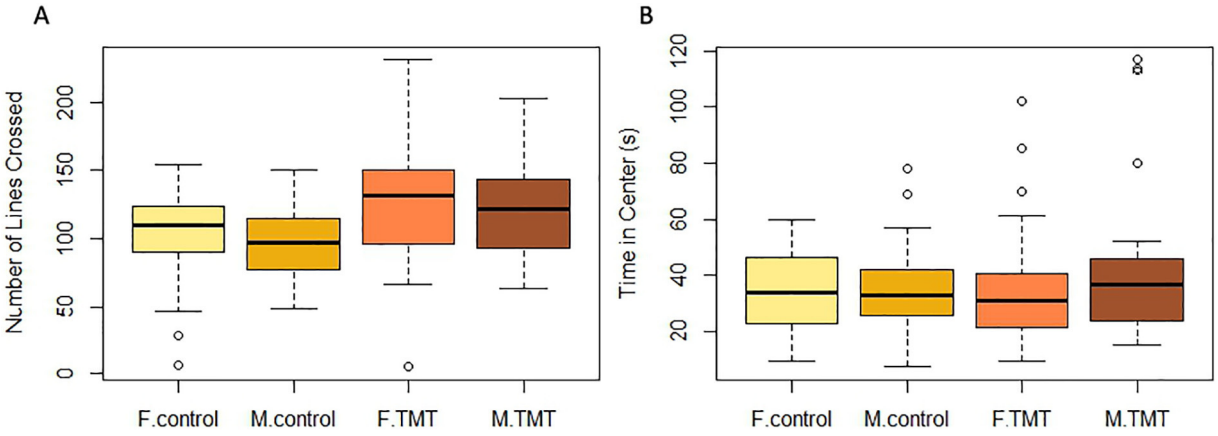

Fig. 2. Juvenile behavior in the open field. A) Female (F) and male (M) juveniles from litters sired by fathers exposed to predator odor (TMT) crossed more grid lines relative to offspring of control males ($p = 0.001$). Within treatment groups, males were less active than females ($p < 0.001$). B) Offspring of TMT-exposed males spent more time in the center relative to control offspring ($p < 0.001$), and sons of TMT-exposed males spent the most time in the center overall ($p = 0.001$). Boxplots include average and interquartile range. Sample sizes, # litters$^{\#\ individuals}$: control females $n = 10^{35}$, control males $n = 9^{17}$, TMT females $n = 15^{48}$, TMT males $n = 14^{29}$.

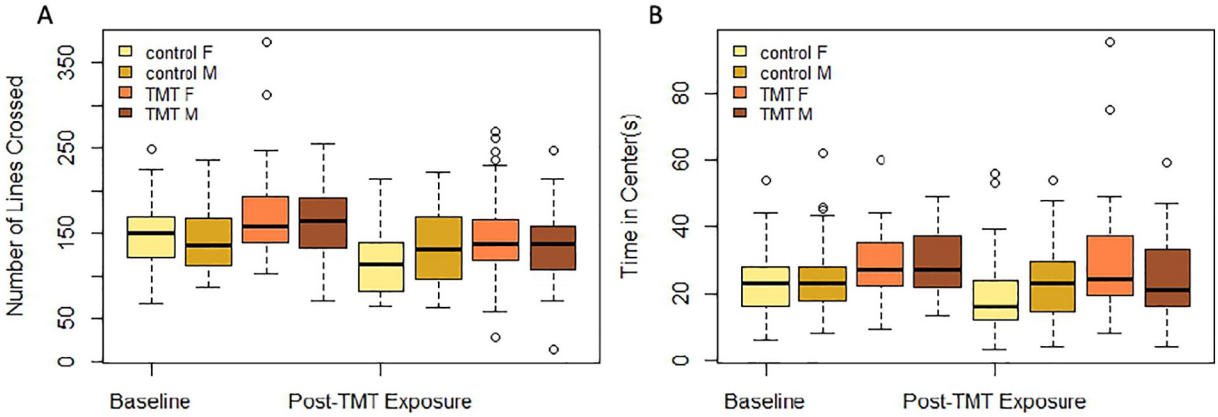

Fig. 3. Adult behavior in the open field. A) Number of lines crossed and B) time in center before and after predator cue (TMT) exposure. A) Before stress, offspring of TMT-exposed males crossed more lines than control offspring ($p = 0.042$). Within groups, females (F) crossed more lines than males (M; $p < 0.001$). After stress, offspring of TMT-exposed males crossed more lines relative to controls ($p = 0.025$) and males were more active than females ($p = 0.043$). The sex specific effect was treatment dependent: sons of control males crossed more lines than daughters, but there was not an effect of sex on offspring of TMT-exposed males ($p < 0.001$). B) Before stress, offspring of TMT-exposed males tended to spend more time in the center of the apparatus relative to offspring of control males ($p = 0.069$). Following stress, offspring of TMT-exposed males spent more time in the center ($p = 0.0067$). Female offspring spent more time in the center than males ($p = 0.02$), but this effect was driven by female offspring of TMT-exposed males, who spent the most time in the center relative to other groups ($p = 0.024$). Error bars, ± 1 SE of mean. Sample sizes, # litters$^{\#\ individuals}$: control females $n = 11^{37}$, control males $n = 9^{23}$, TMT females $n = 18^{52}$, TMT males $n = 16^{37}$.

-18.42 ± 018.74, $t = -0.98$, $p = 0.33$, $R^2c = 1.0$, Fig. 4B), nor was there an effect of sex (LMM, estimate ± SE: -27.61 ± 18.32, $t = -2.25$, $p = 0.13$, $R^2c = 1.0$, Fig. S8).

3.8. Neural gene expression

Offspring of males that experienced predator stress prior to mating had increased relative mRNA expression of mineralocorticoid receptor, *Nr3c2* (ANOVA, $F_{1,19} = 10.53$, $p = 0.0045$, $\eta^2 = 0.37$), and *Bdnf* in the prefrontal cortex (ANOVA, $F_{1,19} = 7.31$, $p = 0.015$, $\eta^2 = 0.29$, Fig. 5B and F). The *Bdnf* result was not significant after Bonferroni correction and *Bdnf* and *Nr3c2* expression did not differ between groups in hippocampus, hypothalamus, or amygdala (ANOVA, all $p \geq 0.3$, Fig. 5B and F). There was a treatment by sex interaction for *Drd1* expression in the prefrontal cortex: daughters of TMT-exposed males had decreased expression relative to other groups (ANOVA, $F_{1,19} = 5.216$, $p = 0.036$, Fig. 5D; see Fig. S9 for all results split by sex). However, this interaction was not significant after Bonferroni correction, and *Drd1* expression did not differ in other brain regions (Fig. 5C). There was no effect of paternal treatment or sex on the relative expression of *Drd2* or *Nr3c1* in any brain region (ANOVA, all $p \geq 0.1$, Fig. 5A and E).

4. Discussion

In this study, we tested for effects of chronic paternal predation stress on offspring behavioral, hormonal, and neural phenotypes. We hypothesized that offspring of males chronically exposed to predator cues prior to mating would exhibit phenotypes associated with predator avoidance (e.g., St-Cyr et al., 2018) and resistance to chronic stress in a predator-rich environment. Specifically, we predicted that offspring of predator cue-exposed males would exhibit 1) reduced activity and time in the center the open field, and 2) a more efficient stress response evidenced by higher plasma CORT following restraint stress and CORT values closer to baseline at recovery relative to control offspring. We also predicted higher glucocorticoid receptor mRNA expression in the hippocampus and prefrontal cortex, facilitating more efficient negative feedback regulation of the acute stress response. None of these predictions were supported. Strikingly, the effect of paternal treatment on

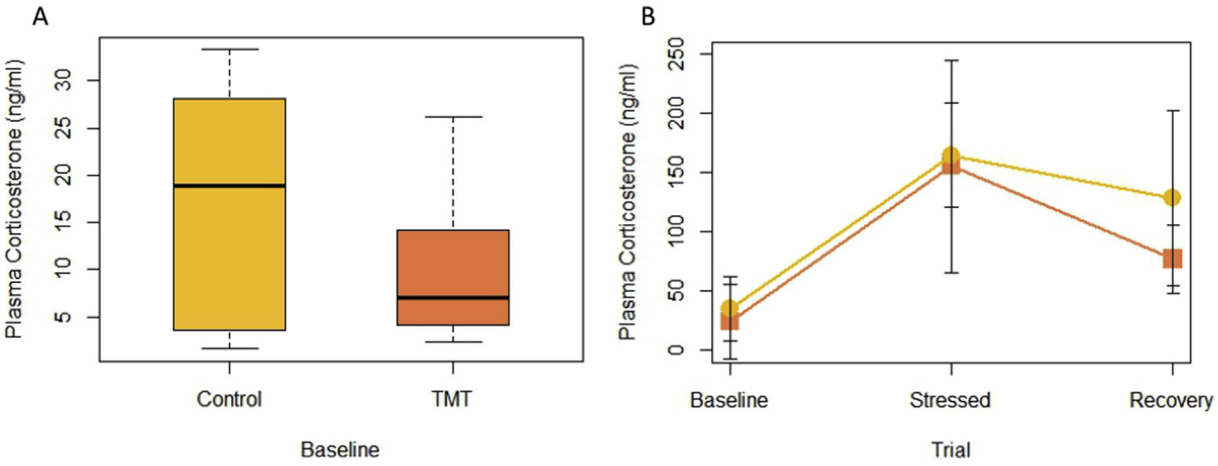

Fig. 4. Effects of paternal predator cue exposure on offspring plasma corticosterone A) at baseline and B) at baseline, during, and after restraint stress. A) Offspring of fathers exposed to predator odor (TMT) had reduced baseline plasma corticosterone relative to control offspring ($p = 0.026$). B) There was no overall effect of paternal treatment on offspring plasma CORT across the three time points. Boxplot in A) includes mean and interquartile range. Error bars in B), ± 1 SE of mean, control offspring indicated with gold circles, offspring of TMT-exposed fathers indicated with orange squares. Sample sizes (litters, sexes combined): baseline, $n = 15$ experimental and $n = 9$ control; stressed $n = 13$ experimental and $n = 10$ control; recovery, $n = 14$ experimental and $n = 11$ control.

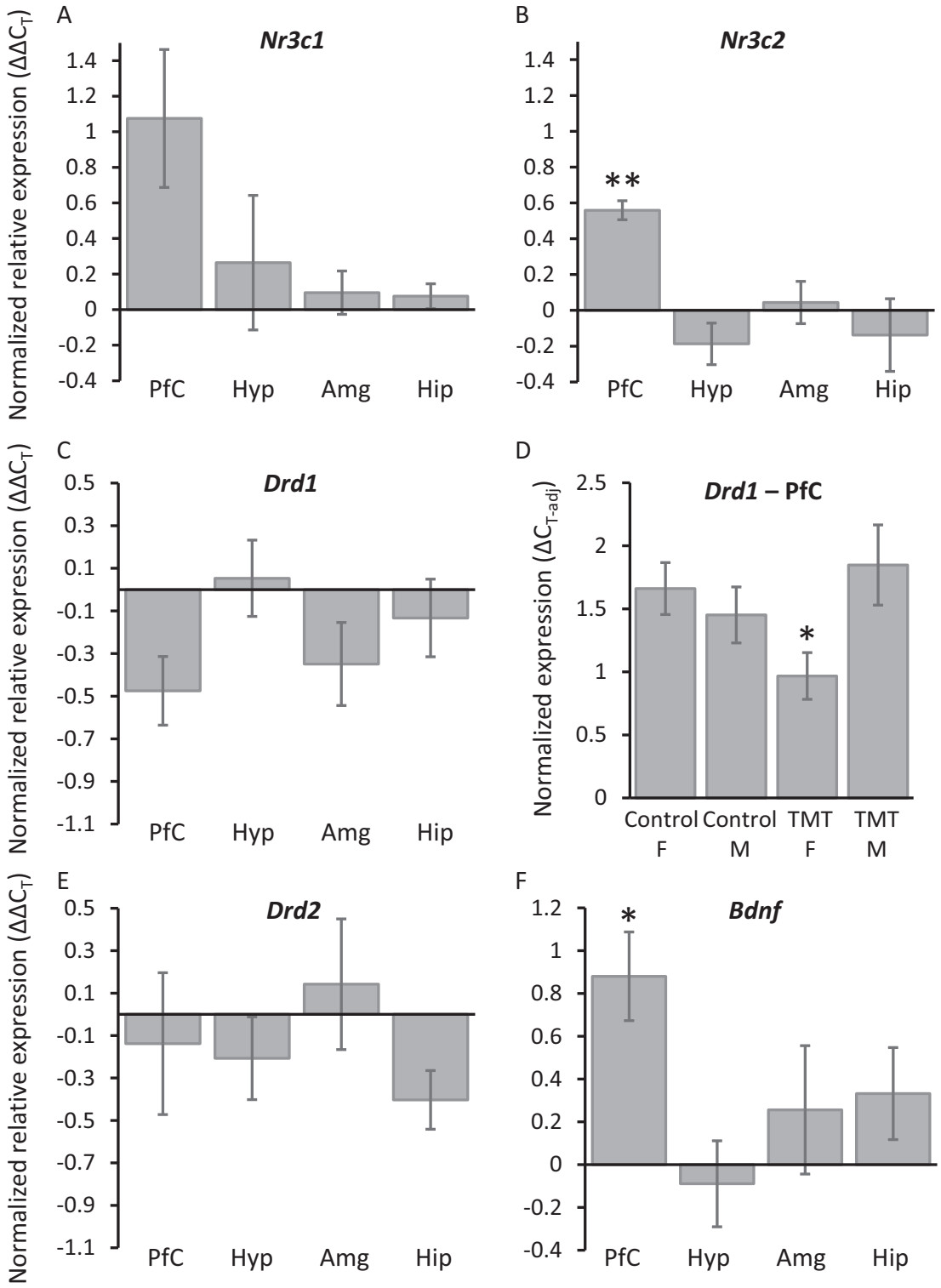

Fig. 5. Relative expression of stress-associated genes in the adult forebrain. A–C and E–F show normalized expression differences ($\Delta\Delta C_T$) in experimental relative to control offspring. Positive values indicate higher and negative values indicate lower expression in experimental offspring. D) Normalized *Drd1* expression in the prefrontal cortex (PfC) for both offspring groups split by sex. Offspring of predator odor-exposed males had higher *Nr3c2* expression ($p = 0.0045$) and higher *Bdnf* expression ($p = 0.015$) in the prefrontal cortex. Daughters of predator odor-exposed males (TMT F) had lower *Drd1* expression ($p = 0.036$) in the prefrontal cortex relative to all other groups. * significant at $\alpha = 0.05$, ** significant at Bonferroni $\alpha = 0.0125$.. Error bars ± 1 SE of mean $\Delta\Delta C_T$ (A-C, E-F) or mean ΔC_T (D). ΔC_{T-adj}, all group means +11 to produce positive values. All genes and brain regions: $n = 5$/sex/treatment. Hyp, hypothalamus; Amg, amygdala; Hip, hippocampus.

open field behaviors was consistently opposite to that predicted. While there was no overall effect of paternal treatment on plasma CORT immediately following stress or after recovery, baseline CORT was lower in the offspring of TMT-exposed males. Glucocorticoid receptor expression was unaffected but prefrontal cortex mineralocorticoid receptor expression was higher in experimental relative to control offspring. We also found evidence of sex-specific effects of paternal treatment. Relative to daughters of TMT-exposed fathers, sons spent

more time in the center of the open field as juveniles but less after exposure to predator odor as adults, and the adult daughters of TMT-exposed males had marginally lower *Drd1* expression in the prefrontal cortex relative to other groups. Given the strong effect of paternal treatment on behavior in the open field, the lack of an effect on stress reactivity was noteworthy. We discuss alternative mechanistic interpretations of these results and consider their ecological implications.

4.1. Paternal effects on offspring behavior and stress reactivity

We found that the offspring of predator cue-exposed fathers were more active in the open field, spent more time in the center of the apparatus, and that activity was not a strong predictor of time in center, indicating that time in center is a largely distinct behavior and not a secondary consequence of activity. Moreover, these behavioral patterns were stable from weaning to adulthood. The OFT has been used to measure anxiety-like behavior in rats and mice for close to a century (Hall and Ballachey, 1932; Korgan et al., 2016); the traditional interpretation of our results is that the offspring of predator cue-exposed males exhibit reduced anxiety-like behavior relative to control offspring. However, the test does not incorporate a specific measure of anxiety and some have argued that it provides and index of fear or risk-aversion rather than anxiety (Gould et al., 2009; Ennaceur, 2014). Thus, and additional test for anxiety-like behavior (e.g. the elevated plus maze) would strengthen our inferences based on the OFT. These caveats notwithstanding, the consistent direction of paternal effects on offspring exploratory behavior and time spent in the center of the open field suggests that paternal predation stress has a protective effect on offspring behavioral response to a non-acute stressor (i.e. a novel, unprotected environment).

Our results are broadly consistent with the effects of paternal predator odor exposure in rats, which include increased boldness in juvenile play and reduced anxiety-like behavior in the elevated plus maze in females (Korgan et al., 2016). Similarly, in lines of mice exposed to paternal postnatal trauma (early maternal separation), offspring and grand-offspring of traumatized fathers exhibited reduced aversion to open space relative to controls, and increased risk-taking in the elevated-plus maze that persisted in the fourth generation (van Steenwyk et al., 2018). In contrast, offspring of male mice subjected to chronic defeat stress exhibited elevated depressive and anxiety-like behaviors (Dietz et al., 2011) and rats whose mothers, fathers, or both parents, were chronically exposed to a live cat prior to mating exhibited more anxiety-like behavior in the elevated plus maze (Azizi et al., 2019). Moreover, the sons of cat-exposed fathers displayed elevated anxiety-like behaviors following exposure to the same predator (Azizi et al., 2019) whereas, in our study, paternal predator odor exposure did not significantly influence the change in offspring open field behavior following exposure to the same predator cue. Taken together these results indicate that, while paternal stress can have profound behavioral effects on offspring and subsequent generations, the direction of these effects may depend on the severity of the stressor experienced by fathers.

In contrast, we found no evidence that paternal predation stress impacts the intensity or efficiency of offspring response to acute stress, as measured by plasma CORT levels immediately post-restraint stress and after 1 h. recovery. Given that restraint elicits an acute stress response in rodents, regardless of parental or developmental experience (Buynitsky and Mostofsky, 2009), one interpretation of this result is that effects of paternal stress on offspring HPA axis function are masked under acutely stressful conditions. Even in maternal predation stress paradigms, in which mothers are exposed to the stressor during pregnancy, effects of maternal experience on offspring CORT response to restraint stress are detected in some but not all studies (St-Cyr and McGowan, 2015; Brachetta et al., 2018). An alternative interpretation is that the paternal predation stress paradigm used here influences aspects of how offspring respond to their environment that are not under the direct influence of the HPA axis. Discriminating between these alternatives would require measuring offspring CORT response to stressors that elicit less extreme responses than restraint. Reduced baseline plasma CORT in offspring of predator cue-exposed males was noteworthy but similarly hard to interpret because the measure was taken at just one time point in the 24 h. cycle. While this phenotype could reflect a dampening effect of increased mineralocorticoid receptor expression on HPA activation (discussed below), we cannot exclude the possibility that the circadian pattern of CORT release is dysregulated in the offspring of predator cue-exposed males. Interestingly, abnormal circadian CORT profiles are associated with PTSD (Dayan et al., 2016; Steudte-Schmiedgen et al., 2016), a disorder that is modeled in rodents exposed to predation stress (Bhattacharya et al., 2019).

We note that, under natural mating conditions, paternal effects are unlikely to act in isolation (Crean and Bonduriansky, 2014; Braun and Champagne, 2014). The compensation hypothesis proposes that, when paired with a low-quality male, females increase their investment in offspring to alleviate paternally-derived disadvantages (Gowaty et al., 2007). A recent study provides direct evidence that this is the case: female mice that were implanted with embryos sired by food-deprived males produced offspring with growth deficits and depression-like behaviors, whereas offspring conceived from natural matings with food-deprived males did not exhibit these deficits (Mashoodh et al., 2018). Thus, the negative effects of paternal food deprivation were mitigated when females had the opportunity to interact with the sires of their offspring (Mashoodh et al., 2018). Although predation stress does not impact male condition in the way that food deprivation does, female rats show less interest in predator odor-exposed males 17 days after the males' last exposure (Korgan et al., 2016). Determining whether maternal compensation contributes to the effect of paternal predation stress on offspring phenotypes reported herein would require comparison between artificially implanted and naturally mated females.

4.2. Paternal effects on offspring neural gene expression

Normal HPA axis function relies on corticosteroid binding to both glucocorticoid and mineralocorticoid receptors. Whereas glucocorticoid receptor binding in the hippocampus and prefrontal cortex is critical to negative feedback regulation of the acute stress response, mineralocorticoid receptors in these brain regions are more important to allostatic regulation of glucocorticoid levels under non-stressed conditions (McKlveen et al., 2015; de Kloet et al., 2019). Experimental manipulation of corticosteroid receptor expression or function indicates that mineralocorticoid receptors in the forebrain have an inhibitory effect on HPA axis activity and determine the threshold at which an acute stress response is launched (de Kloet et al., 2019). For example, reduced *NR3C2* expression in humans is associated with increased vulnerability to depression (Kuningas et al., 2007) and higher stress reactivity (DeRijk et al., 2006), while mineralocorticoid receptor antagonists increase anxiety-like behaviors and both basal and stress-induced CORT levels in rats (Dallman et al., 1989; Ratka et al., 1989; Chen et al., 2019). Conversely, higher forebrain *Nr3c2* expression is associated with lower anxiety-related behaviors and enhanced stress-coping in mice (Veenema et al., 2003; Lai et al., 2007; Rozeboom et al., 2007). In our study, offspring of predator cue-exposed males had higher *Nr3c2* expression in the prefrontal cortex relative to control offspring. Given the anxiolytic effects of increased mineralocorticoid receptor expression in the forebrain, this neural phenotype is consistent with evidence for reduced anxiety-like behavior in the offspring of predator cue-exposed males.

We also found evidence for increased *Bdnf* expression in offspring sired by TMT-exposed males vs. control offspring, and reduced *Drd1* expression in the daughters of TMT-exposed males. Although the effects of paternal treatment on *Bdnf* and *Drd1* expression did not survive Bonferroni correction, it is noteworthy that evidence for altered expression in the offspring of predator cue-exposed males was exclusive to the prefrontal cortex. Acting above the level of the HPA axis, the

prefrontal cortex coordinates stress response circuitry and is particularly important to stress adaptation, both minimizing stress reactivity and protecting against chronic stress (McKlveen et al., 2015; de Kloet et al., 2019). The prefrontal cortex is also highly sensitive to the damaging effects of chronic stress (Arnsten, 2009). Under the match-mismatch hypothesis (reviewed in Schmidt, 2011), paternal perception of high predation risk should have a protective effect on the brains of offspring chronically exposed to the same stressor. It will be important to determine whether evidence for paternal buffering under mildly stressful conditions (i.e. the OFT) can be extended to chronic stress in the offspring of predator cue-exposed males.

We note two caveats to the neural expression data. First, the four brain regions in our analysis are subdivided into nuclei or subregions with distinct patterns of connectivity and, particularly in the case of the hypothalamus, discrete functions. It is possible that finer dissections within each region would reveal effects on expression that are undetectable here. Second, the candidate gene approach provides no information about downstream effects of altered expression. This is particularly relevant for genes such as cytoplasmic steroid hormone receptors, whose ligand-bound products translocate to the nucleus and affect the transcription of multiple target genes (Robert-Nicoud et al., 2001; Meijer et al., 2019). Given the effect of paternal treatment on *Nr3c2* expression in prefrontal cortex, whole transcriptome analysis of expression in this brain region would be of particular interest.

4.3. Ecological implications of paternal effects on offspring behavior

Our original hypothesis, that offspring of predator cue-exposed males would show increased sensitivity to predator odor, reduced activity and increased avoidance of open areas, was based on the assumption that a stress-reactive and risk-averse phenotype should benefit a prey species in a high predation environment. However, this hypothesis did not take into account potential trade-offs between predator avoidance and other activities such as foraging and reproduction. Whereas it is advantageous for prey animals to avoid predators, launching a stress response every time a predator cue is detected is costly, especially in predator-rich areas. Thus, predator-rich environments may favor more stress-resilient animals (Fisher et al., 2014; Orrock and Fletcher, 2014; Chaby et al., 2015). Indeed, behavioral studies in wild rodent populations suggest that both familiarity with the predator (Abom and Schwarzkopf, 2016) and increased predator density (Orrock and Fletcher, 2014) promote higher activity and more risk-taking behavior (e.g. foraging in open areas). Given that fathers in the present study were chronically exposed to ecologically relevant doses of predator odor (Buron et al., 2007), it is possible that increased activity and time spent in the center of the open field are indicative of offspring behaviors that would be adaptive in a predator-dense environment.

We also found that adult daughters of TMT-exposed fathers spent more time in the center of the open field following TMT exposure than any other group. Moreover, these females were the only group that did not reduce time in the center post-TMT exposure relative to baseline measures (Fig. 3B). These results are interesting when considered from the perspective of mammalian sex differences in dispersal and reproductive investment. Like most mammals, female house mice are typically philopatric whereas males typically disperse from their natal site (Gerlach, 1990; Pocock et al., 2005; Mabry et al., 2013). Thus, the probable match between parent and offspring environments is higher for fathers and daughters than for fathers and sons. Moreover, given the high metabolic costs of gestation and lactation (Speakman, 2008), females may benefit more than males from engaging in risk-taking behaviors that allow them to forage more efficiently.

5. Conclusions

A growing body of work demonstrates the importance of paternal effects as modulators of offspring behavior, physiology, and disease risk (Crean and Bonduriansky, 2014; Yeshurun and Hannan, 2019). Whereas ground-breaking work on germline transmission of paternal experience used acute or chronic variable stressors (Rodgers et al., 2013; Dias and Ressler, 2014), our study contributes to understanding of the intergenerational effects of paternal exposure to less severe but ecologically relevant stressors. The offspring of fathers exposed to predator odor prior to mating exhibited more exploratory and less anxiety-like behaviors, and had lower baseline plasma corticosterone and higher mineralocorticoid receptor expression in the prefrontal cortex but did not differ from control offspring in their acute stress response. These results suggest that fathers exposed to predation stress produce offspring that are buffered from non-acute stressors and, potentially, better adapted to a high predation environment. Future work will investigate the underlying epigenetic mechanisms, for which paternal sperm microRNAs are strong candidates (Rodgers et al., 2013, 2015; Short et al., 2016; Conine et al., 2018). Importantly, this study provides evidence that ecologically relevant parental experience can be transmitted through the paternal germline and can exert consistent effects on offspring phenotypes throughout development.

Declaration of competing interest

None.

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Appendix A. Supplementary tables and figures

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