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Dysregulation of neonatal hippocampal cell genesis in the androgen insensitive Tfm rat

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ABSTRACT

The first two weeks of life are a critical period for hippocampal development. At this time gonadal steroid exposure organizes sex differences in hippocampal sensitivity to activational effects of steroids, hippocampal cell morphology and hippocampus dependent behaviors. Our laboratory has characterized a robust sex difference in neonatal neurogenesis in the hippocampus that is mediated by estradiol. Here, we extend our knowledge of this sex difference by comparing the male and female hippocampus to the androgen insensitive testicular feminized mutant (Tfm) rat. In the neonatal Tfm rat hippocampus, fewer newly generated cells survive compared to males or females. This deficit in cell genesis is partially recovered with the potent androgen DHT, but is more completely recovered following estradiol administration. Tfm rats do not differ from males or females in the level of endogenous estradiol in the neonatal hippocampus, suggesting other mechanisms mediate a differential sensitivity to estradiol in male, female and Tfm hippocampus. We also demonstrate disrupted performance on a hippocampal-dependent contextual fear discrimination task. Tfm rats generalize fear across contexts, and do not exhibit significant loss of fear during extinction exposure. These results extend prior reports of exaggerated response to stress in Tfm rats, and following gonadectomy in normal male rats.

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Introduction

Gonadal steroid exposure during early postnatal development induces enduring sex differences in hippocampal sensitivity to gonadal steroids in the adult, as measured by both cell morphology and behavior (e.g., Bangasser and Shors, 2007; Isgor and Sengelaub, 2003). In other brain regions, testosterone and its aromatized product estradiol mediate sex specific cell survival and death to produce the sexual dimorphisms in cell number and volume in areas directly mediating sex behavior and gonadotropin secretion (McCarthy, 2008; Morris et al., 2004). In the sexually dimorphic nucleus of the preoptic area (SDN-POA), and the nearby anteroventral periventricular nucleus (AVPV), neonatal males and females initially have the same number of cells. However, higher estradiol in males reduces cell death in the SDN-POA (Davis et al., 1996) but induces apoptosis in the AVPV (Waters and Simerly, 2009). These brain regions directly support reproductive physiology and behavior and the sex differences in the size of the nuclei are due to cell survival, rather than cell genesis and thus differential apoptosis was considered the dominant mechanism by which sex differences in the brain are established. We recently reported higher rates of cell genesis in the male neonatal hippocampus compared to females (Bowers et al., 2010; Zhang et al., 2008), an area of the brain not involved in reproduction but instead critical for learning, memory and modulation of the stress axis.

The sex difference in hippocampal cell genesis is modulated by hormones since administration of estradiol or the potent non-aromatizable androgen dihydrotestosterone (DHT) to neonatal females increases hippocampal cell genesis to that of males, although neither treatment increases cell genesis in the male hippocampus (Bowers et al., 2010; Zhang et al., 2008). Conversely, treatment of males with either an estrogen receptor (ER) antagonist or aromatase inhibitor drastically reduces levels of cell genesis to below that of females while antagonism of estrogen signaling has no effect on cell genesis in the developing female hippocampus (Bowers et al., 2010). However, despite the clear role of estradiol in neonatal hippocampal cell genesis, there is no detectable sex difference in endogenous hippocampal estradiol levels at birth, suggesting estradiol alone is not organizing the male hippocampus, and that there are important sex differences in sensitivity to estradiol (Amateau et al., 2004; Konkle and McCarthy, 2011). While it is clear that endogenous estradiol is critical for basal rates of cell genesis in males but not

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females, there is also a poorly understood role for androgens in hippocampal development.

Both androgen receptors (AR) and estrogen receptor beta (ERbeta) are expressed in neural precursors harvested from embryonic and adult mouse brain (Brännvall et al., 2002, 2005). Whether proliferating cells in the developing hippocampus express androgen receptors is currently unknown. Estrogen receptors alpha (ERα) and ERβ are expressed in the perinatal hippocampus, with higher expression in the male postnatal hippocampus (Ivanova and Beyer, 2000). Expression of ERα increases sharply on postnatal day 4 (PN4) in pyramidal cells of CA1 and CA3 (O'Keefe and Handa, 1990; O'Keefe et al., 1995; Solum and Handa, 2001). Less is known about the contribution of ERβ to hippocampal development, but analysis of ERβ knockout mice suggests that this receptor plays a role in development and migration of calretinin-positive GABAergic interneurons (Fan et al., 2006).

In the adult hippocampus androgens and estrogens impact cell genesis, morphology and excitability in a sex-specific manner (Barker and Galea, 2008; Estrada et al., 2006; Foradori et al., 2007; Gould et al., 1990; Huang and Woolley, 2012; MacLusky et al., 2004, 2006; Morris et al., 2005; Spritzer and Galea, 2007; Woolley and McEwen, 1992). Androgen receptors are expressed in the hippocampus, with higher expression in pyramidal neurons of CA1 and CA3 relative to the dentate gyrus (Brännvall et al., 2005; Kerr et al., 1995; Tabori et al., 2005; Xiao and Jordan, 2002). Astrocytes in all subregions of the adult hippocampus also express androgen receptors (Tabori et al., 2005). Androgens, but not estrogens, are necessary for maintenance of dendritic spines on pyramidal neurons of the adult male hippocampus (Leranth et al., 2003; Maclusky et al., 2006). Gonadectomy of adult males also reduces survival of adult generated granule cells in the dentate gyrus (Spritzer and Galea, 2007). This is reversed by treatment with testosterone or DHT but not estradiol (Spritzer and Galea, 2007) while estradiol does increase cell genesis in the hippocampus of adult ovariectomized female rats (Mazzucco et al., 2006), suggesting androgens and estrogens play differential roles in neuronal survival versus proliferation, respectively.

The testicular feminization mutation (Tfm) is a naturally occurring point mutation of the gene encoding the androgen receptor, rendering the Tfm male rat insensitive to physiological levels of androgens (Langley et al., 1998; Yarbrough et al., 1990). As a result, Tfms present with feminized genitalia and exhibit deficits in some aspects of masculine sex behavior (Hamson et al., 2009; Olsen and Whalen, 1981). Gonadal steroid synthesis is intact in the Tfm male so that testosterone is relatively high, but within the normal range (Roselli et al., 1987) and estrogen binding in the adult Tfm brain also appears to be normal (Olsen and Whalen, 1982). Supraphysiological doses of DHT are capable of activating the mutated androgen receptor in Tfm male rats (Langley et al., 1998; Yarbrough et al., 1990). To our knowledge, ER and AR levels in the developing or adult Tfm male brain have not been compared to normal males or females.

Androgen insensitivity results in an exaggerated stress response in Tfm males, suggesting that once activated, inhibition of the stress axis is disrupted in these animals (Zuloaga et al., 2011a, 2011b). Inhibition of the stress axis relies in part on the hippocampus (Jankord and Herman, 2008). In the experiments presented here, we characterize cell genesis in the neonatal Tfm hippocampus, a critical developmental period of hippocampal sexual differentiation and development. This developmental period is a time at which gonadal steroids contribute to sexual dimorphisms in the stress axis and hippocampal responses to stress and learning (Bangasser and Shors, 2007; Shors et al., 2004). We further characterize the adult Tfm male in a hippocampal-dependent contextual fear discrimination task to determine whether androgen insensitivity exacerbates contextual fear (Antoniadis and McDonald, 2000; Frankland et al., 1998). Our results implicate androgen sensitivity in postnatal hippocampal development and hippocampal-dependent expression of stress and fear.

Material and methods

Animals

Tfm Long Evans carrier dams were identified by PCR as previously described (Fernandez et al., 2003). Wild type (WT) male, female and Tfm male pups were generated in a breeding colony established at the University of Maryland School of Medicine from founders generated at Michigan State University. The day of birth was defined as postnatal day 0 (PN0). Rats were housed under a 12:12 h light:dark cycle, with food and water freely available. All procedures were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Hormone and BrdU administration

For Experiment 1A, pups were injected intraperitoneally with 50 μg BrdU (50 μg in 0.1 ml of 0.9% saline) each morning of PN0–PN3 and euthanized on PN4, 24 h after the last BrdU injection. For Experiment 1B, pups were injected intraperitoneally with BrdU (100 μg in 0.1 ml of 0.9% saline) and euthanized either 2 or 6 h later. For Experiments 3 and 4, WT male, female and Tfm male rat littermates were randomly assigned to a hormone treatment condition. Pups were subcutaneously treated with either estradiol benzoate (Sigma; 100 μg/0.1 ml in sesame oil), dihydrotestosterone (Sigma; 100 μg/0.1 ml in sesame oil) or vehicle alone (0.1 ml sesame oil) the morning of birth (PN0). Two hours after steroid or oil vehicle injections, all pups were injected with BrdU (100 μg in 0.1 ml of 0.9% saline) to label mitotic cells. Steroid doses were based on ability to masculinize the neonatal female brain and BrdU dose was based on ability to saturate all dividing cells without inducing cell toxicity and potentially death (Bowers et al., 2010; Zhang et al., 2008).

Tissue collection and cell quantification

Pups were deeply anesthetized with sodium pentobarbital (250 mg/kg) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) on P0, P1 or P30. Brains were removed and fixed for 48 h in 4% PFA and then stored in 30% sucrose in PBS before 45 μm coronal sections were taken on a cryostat. Given the rostrocaudal extent of the hippocampus in P0, P1 and P4 brains, a 1 in 8 series generated 6 sections for cell density analysis. For analysis of P30 brains, a 1 in 12 series generated 10 sections containing the hippocampus for cell density analysis. Cell density for each section and subregion was determined using Neurolucida software. These cell counts were collected from six 100 × 100 μM counting frames within the principal cell layer of the dentate gyrus, CA1 and CA3, avoiding cells in the outermost focal planes. The totals for each section and subregion were averaged for each animal.

Immunohistochemistry

Free-floating sections were rinsed with 0.1 M phosphate buffered saline (PBS) and incubated with 3% hydrogen peroxide in PBS for 30 min. For BrdU immunohistochemistry, tissue was incubated in 2 N hydrochloric acid (HCl) for 30 min at 37 °C to denature DNA. Sections were then rinsed in borate buffer followed by PBS rinses. Sections were incubated in 5% goat serum in PBS with 0.4% Triton X-100 (PBS-T) for 60 min, and then incubated with a monoclonal antibody against BrdU (1:10,000, Caltag Laboratories, CA, USA) at room temperature for 60 min and then overnight at 4 °C. Following PBS rinses, sections were incubated in biotinylated

antimouse secondary antibody in PBS-T (1:1000, Vector, Auckland, New Zealand) rinsed with PBS and incubated in Vectastain Elite ABC (1:1000, Vector). BrdU-immunoreactive (BrdUir) cells were visualized with diaminobenzidine (DAB). For Ki-67, sections were treated as described above with the omission of HCl incubation. Tissue was incubated in a polyclonal antibody against Ki67 in PBS-T (1:250, Millipore, Darmstadt, Germany) for 60 min at room temperature and then 4 °C for 48 h followed by biotinylated anti-rabbit secondary antibody (1:1000). Ki67 immunoreactivity was also detected with DAB.

Radioimmunoassay for estradiol

On PN0, hippocampi from male, female and Tfm rats were collected. Hippocampi were mechanically homogenized in a cold lysis buffer containing 0.1% protease inhibitor. Protein content was determined by the Bradford assay. Following ether and methanol extraction of nonconjugated estradiol, the extract was reconstituted in estradiol calibrator (DSL 4401). A 25 µl aliquot of each sample was diluted in 475 µl of 0.1 M PBS and sent to the Ligand Assay and Analysis Core Facility at the University of Virginia Center for Research in Reproduction (University of Virginia, Charlottsville, VA) for RIA using commercially available kits for estradiol (DSL-39100) with a sensitivity of 0.6 pg/ml. Estradiol was calculated and analyzed as pg/mg protein.

Fear conditioning

Tfm and wild-type male Long Evans rats were 60–90 days of age at the onset of the experiment. Contextual fear was measured using San Diego Instruments Freeze Monitor software. Suppression of activity, or freezing, was indexed by the amount of time taken for the animal to break 3 infrared beams in 5 s intervals. For each 5 s interval, the amount of time taken to break the third beam was divided by the total 5 s to calculate percent time freezing. If the third beam is not broken in the 5 s interval, then the percent time spent freezing for that interval is 100%. Use of 5 s intervals has been demonstrated to be as accurate as scoring freezing using manual time-sampling techniques (Valentinuzzi et al., 1998). The percent time freezing was then averaged over 2-min intervals for each animal.

To promote acquisition of context discrimination, rats were given a 10 min exposure in each context on 3 consecutive days prior to fear conditioning, during which baseline activity was recorded. For context discrimination, two distinct contexts were created using different levels of illumination and discriminative odors. Context A was scented with Vick's Vap-O-Rub, and Context B with 10% white vinegar. Context A was illuminated by a small key light located in the top of the testing apparatus, while Context B was illuminated by a 40 W bulb in the outer sound attenuating chamber, producing distinct levels of illumination in the conditioning chamber. On the fourth experimental day, rats were placed in only one context. Half of each group received the shock unconditioned stimulus (US) in Context A and half received the US in Context B. Following a 3 min period in which no stimuli were delivered, rats were administered a 0.8 mA, 1 s shock delivered through the floor of the chamber. Two more shocks of the same duration and magnitude were delivered at an inter-shock interval of 2 min ± 1 min. Rats were then tested for freezing in each context for 10 min per session across two consecutive days in a counterbalanced order, with half of each group tested in the no shock context first, and half tested in the shock context first. Each 10 min session was analyzed in five 2 min intervals, representing the percent time spent freezing in each interval.

Results

Experiment 1: Postnatal cell genesis in the male, female and Tfm male rat hippocampus

Male (n = 7), female (n = 7) and Tfm (n = 7) rats were euthanized on PN4, 24 h after the last BrdU injection. One-way ANOVA revealed a significant effect of genotype on the number of BrdUir cells detected in the dentate gyrus ($F[2,18] = 40.98$, $p = 0.0001$, $\eta_p^2 = 0.82$), CA1 ($F[2,18] = 14.411$, $p = 0.0001$, $\eta_p^2 = 0.61$), and CA3 ($F[2,18] = 58.65$, $p = 0.0001$, $\eta_p^2 = 0.86$; Fig. 1). Bonferroni posthoc comparisons confirmed that males exhibited higher numbers of BrdUir cells than females in the dentate gyrus ($p < 0.001$, $d = 3.92$) or Tfm males ($p < 0.001$, $d = 3.68$). Similarly, higher numbers of BrdUir cells were detected in the male CA1 region compared to females ($p < 0.001$, $d = 2.21$) or Tfm males ($p < 0.0001$, $d = 2.43$). The same pattern was detected in CA3. More BrdUir cells were detected in the male CA3 subregion compared to females ($p < 0.001$, $d = 3.61$) and Tfm males ($p < 0.001$, $d = 4.23$). Female and Tfm males did not differ from each other in any of the three subregions ($ps > 0.05$). These results replicate our previous finding of higher BrdU labeling in the postnatal male hippocampus and further implicate a role for androgens as Tfm males do not have masculine levels of cell genesis.

Experiment 2: Cell proliferation in the male, female and Tfm male rat hippocampus

To determine whether the sex difference in cell genesis was due to decreased proliferation in female or Tfm males, pups of each genotype were administered BrdU and sacrificed either 2 h (male, n =6, female, n = 7, Tfm, n = 7) or 6 h (male, n = 8, female, n = 7, Tfm, n = 7) later. A factorial ANOVA with genotype (3) and time (2) as between subjects factors and the number of BrdUir cells as the dependent variable was conducted separately for the dentate gyrus, CA1 and CA3. In the dentate gyrus, this analysis confirmed a significant main effect of genotype ($F[2,36] = 34.37$, $p < 0.001$, $\eta_p^2 = 0.65$) and

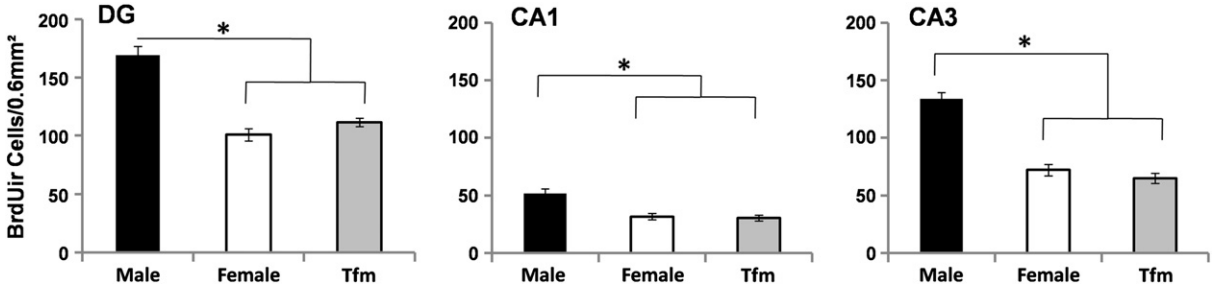

Fig. 1. Wild type male, female and Tfm male pups were treated with 4 injections of BrdU on PN0–PN3. Rats were euthanized 24 h after the last BrdU injection on PN4. Bars (±SEM) represent the average number of cells detected in the principle cell layer of the dentate gyrus, CA1 or CA3. Significantly higher numbers of BrdUir cells were detected throughout the hippocampus of wild type males relative to wild type female or Tfm males.

time ($F[1,36] = 8.51$, $p < 0.006$, $\eta_p^2 = 0.19$) and a genotype × time interaction ($F[2,36] = 11.27$, $p < 0.001$, $\eta_p^2 = 0.38$; Fig. 2). This interaction is due to higher cell numbers at 6 h in WT male and female rats compared to cell numbers detected in WT male and female rats euthanized 2 h after BrdU administration. Numbers of BrdUir cells are not higher at 6 h compared to 2 h in Tfm males.

The pattern of BrdU positive cells in the pyramidal cell layers of CA1 and CA3 differed from that in the dentate gyrus (Fig. 2). In CA1, factorial ANOVA confirmed a significant main effect of genotype ($F[2,36] = 52.17$, $p < 0.0001$, $\eta_p^2 = 0.74$) and time ($F[1,36] = 7.41$, $p < 0.01$, $\eta_p^2 = 0.17$), and a genotype × time interaction ($F[2,36] = 5.09$, $p < 0.01$, $\eta_p^2 = 0.22$). Again, cell numbers detected were higher 6 h after BrdU administration than 2 h after in WT males. Though the number of cells detected 6 h after BrdU administration were only slightly higher than the number detected 2 h after BrdU in separate groups of females, it suggests that more cells labeled by BrdU survive this short interval in both WT males and females than Tfm males. In CA3, factorial ANOVA confirmed a significant main effect of sex ($F[2,36] = 30.46$, $p < 0.001$, $\eta_p^2 = 0.62$) and time ($F[1,36] = 36.69$, $p < 0.0001$, $\eta_p^2 = 0.50$; Fig. 2). There was no interaction between sex and time. Tfm males do not exhibit a reduction in BrdUir cells at 6 h compared to 2 h as in the dentate gyrus but the difference between the timepoints is not as robust as detected in WT males and females across the two timepoints. Together, these results suggest that fewer proliferating cells survive in the developing Tfm hippocampus. To further determine whether proliferation of cells differed between the genotypes, we quantified the number of Ki67ir cells in tissue collected from WT male ($n = 7$), female ($n = 8$) and Tfm males ($n = 6$) on PN1, 24 h after birth (Fig. 2). Ki67 is a marker of cell proliferation that is expressed in all active phases of the cell cycle, whereas BrdU is incorporated into the cell during the S-phase (Gerlach et al., 1997; Scholzen and Gerdes, 2000). One-way ANOVA confirmed a significant effect of genotype on Ki67ir cells in the dentate gyrus ($F[2,18] = 14.70$, $p < 0.0001$, $\eta_p^2 = 0.62$), CA1 ($F[2,18] = 4.40$, $p = 0.028$, $\eta_p^2 = 0.32$), and CA3 ($F[2,18] = 7.68$, $p = 0.004$, $\eta_p^2 = 0.46$). In the dentate gyrus and CA3, WT males had more Ki67ir cells than females ($ps < 0.0001$, $d = 2.4$ and $d = 2.16$ respectively) and Tfm males ($p < 0.0001$, $d = 1.99$ and $d = 1.59$ respectively) but female and Tfm male did not differ from each other. In CA1, Ki67ir cells were higher in males than females ($p < 0.02$, $d = 1.14$), but the number of Ki67ir cells in the Tfm CA1 subregions was intermediate and therefore did not differ from either WT males or females.

Experiment 3: Hormonal modulation of cell genesis in male, female and Tfm male rats

Animals were treated with oil vehicle, DHT or estradiol 2 h prior to a single dose of BrdU on PN0 and euthanized 24 h later to assess the effect of hormone administration on cell proliferation and short term survival (Fig. 3). Male pups were not treated with either DHT or estradiol. The number of rats in each group was: WT males + oil ($n = 7$), females + oil ($n = 8$), females + E2 ($n = 7$), females + DHT ($n = 7$), Tfm + oil ($n = 6$), Tfm + E2 ($n = 7$), Tfm + DHT ($n = 7$). A factorial ANOVA with genotype and treatment as between subjects variables confirmed a significant main effect of genotype ($F[2,42] = 67.177$, $p < 0.0001$, $\eta_p^2 = 0.76$) and treatment ($F[2,42] = 13.66$, $p < 0.0001$, $\eta_p^2 = 0.39$) on the number of BrdUir cells in the dentate gyrus. The genotype × treatment interaction was also significant ($F[2,42] = 3.66$, $p < 0.034$, $\eta_p^2 = 0.14$). There were more BrdUir cells in the WT male dentate gyrus compared to oil-treated females ($p < .0001$, $d = 4.54$), females + E2 ($p < 0.0001$, $d = 2.26$) or females + DHT ($p < 0.0001$, $d = 2.76$). Tfm males treated with oil were lower compared to WT males ($p < 0.0001$, $d = 4.29$). Neither estradiol ($p < 0.0001$, $d = 2.61$) nor DHT ($p < 0.0001$, $d = 3.32$) increased cell genesis in Tfm males to that of WT males. Estradiol increased cell genesis in females ($p < 0.0001$, $d = 5.53$) and Tfm males ($p < 0.03$, $d = 3.09$) at this time point relative to same genotype rats treated with oil. DHT increased BrdUir cells in female ($p < 0.002$, $d = 4.54$) and Tfm males ($p < 0.05$, $d = 2.64$) as well.

In the pyramidal cell layer of CA1, there was a significant effect of genotype ($F[2,42] = 21.13$, $p < 0.0001$, $\eta_p^2 = 0.50$) and treatment ($F[2,42] = 13.03$, $p < 0.0001$, $\eta_p^2 = 0.38$), but no significant interaction between the two. Oil-treated males had more BrdUir cells than either females + oil ($p = 0.0001$, $d = 2.54$) or Tfm males + oil ($p < 0.001$). Estradiol increased the number of BrdUir cells in females compared to females + oil ($p < 0.001$, $d = 5.46$) but not in Tfm males ($p > 0.05$).

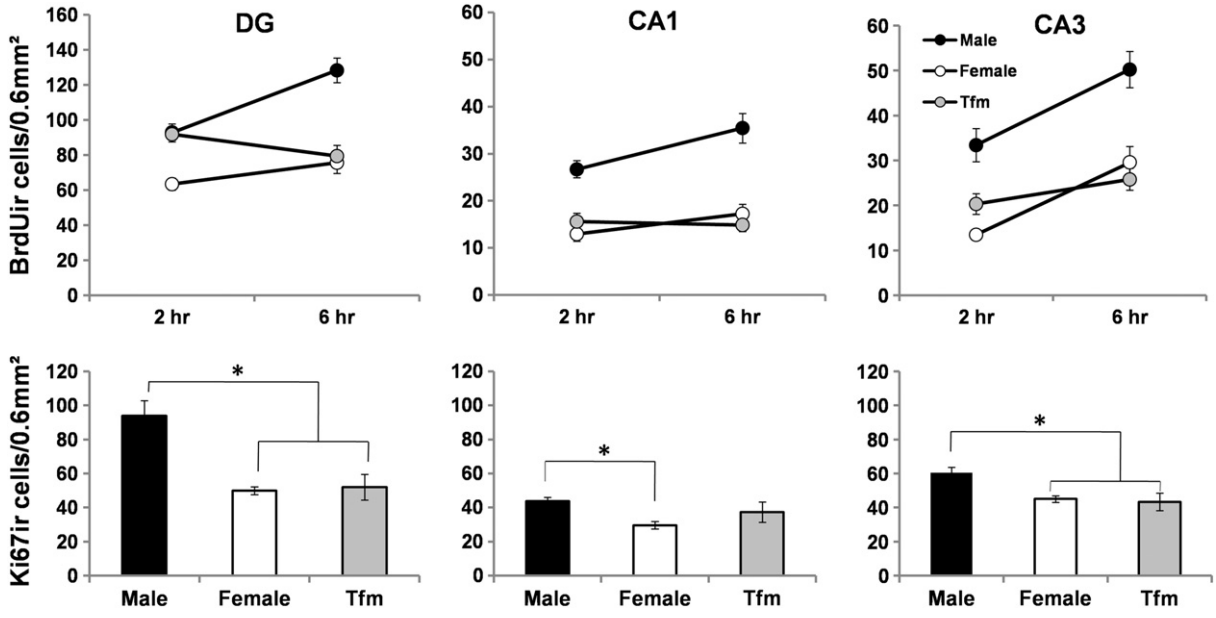

Fig. 2. Top panel: Wild type male, female and Tfm male pups were treated with a single injection of BrdU on PN0 and sacrificed either 2 or 6 h later. Bars (±SEM) represent the average number of BrdUir cells detected in the principle cell layer of the dentate gyrus, CA1 or CA3. Bottom panel: Ki67 immunoreactive cells were quantified in wild type male, female and Tfm male pups euthanized on PN1. Cell proliferation was greater in the dentate gyrus and CA3 subregion relative to both females and Tfm males. In CA1, Tfm males did not differ from wild type male or female rats.

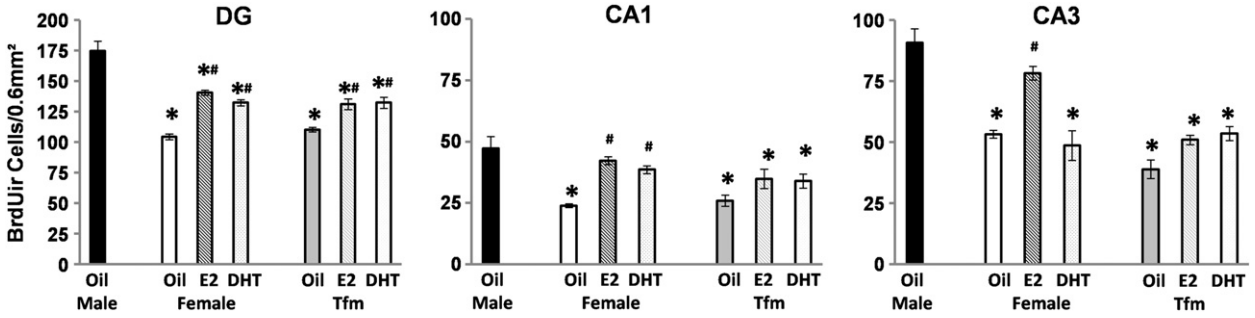

Fig. 3. Wild type female and Tfm male pups were treated with oil vehicle, estradiol or DHT on the morning of birth. Wild type male pups were treated with oil vehicle. Two hours following steroid treatment, male, female and Tfm male pups were treated with a single dose of BrdU (100 mg/kg). All pups were euthanized 24 h following BrdU administration. * indicates significance difference from males; # indicates significant difference from same sex vehicle treated controls.

Similarly, DHT only increased BrdUir cells in females ($p < .0001$, $d = 4.44$). Hormone treated Tfm males did not differ from Tfm oil treated controls ($p > 0.05$).

In the pyramidal cell layer of CA3 there was again a significant effect of genotype ($F[2,42] = 50.76$, $p < 0.0001$, $\eta_p^2 = 0.70$) and treatment ($F[2,42] = 12.28$, $p < 0.0001$, $\eta_p^2 = 0.36$) and in this hippocampal subregion there was a significant interaction between the two variables ($F[2,42] = 8.81$, $p < 0.001$, $\eta_p^2 = 0.29$). WT males again retained more BrdUir cells in CA3 compared to females ($p < .0.0001$, $d = 3.38$) or Tfm males ($p < 0.0001$, $d = 4.07$) and estradiol increased the number of BrdUir cells in females compared to vehicle treated controls ($p < 0.001$, $d = 4.02$) so that estradiol treated females did not differ from WT males. However, at this time point and in this hippocampal subregion, neither DHT nor estradiol significantly increased the number of BrdUir cells in the Tfm male hippocampus compared to vehicle treated Tfm control rats (ps > 0.05). As a result, Tfm males treated with estradiol or DHT remained significantly different from WT males ($p < 0.0001$, $d = 3.40$ and 3.12 respectively).

Since Tfm males responded to estradiol with increased cell proliferation in the dentate gyrus, we measured estradiol in the hippocampus at PN0 to determine whether Tfm males are deficient in estrogen levels compared to male and female rats. Previous reports have found decreased aromatase activity in the Tfm brain, which could reduce the amount of estradiol produced (Roselli et al., 1987). Radioimmunoassay for endogenous estradiol in the whole hippocampus revealed no significant differences in the neonatal WT male, female and Tfm male hippocampus on PN0. The mean ($\pm$SEM) concentration of estradiol, quantified as pg/mg of protein was: male ($n = 8$) 1.99 $\pm$ .0.39, female ($n = 8$) 1.45 $\pm$ 0.27; and Tfm male ($n = 7$) 1.75 $\pm$ 0.16. This result replicates the absence of a sex difference between males and females, and the level of neonatal hippocampal estradiol previously reported by our laboratory in Sprague Dawley rats using the same extraction protocol as here (Konkle and McCarthy, 2011).

Experiment 4: Hormonal modulation of cell survival in male, female and Tfm male rats

Survival of cells generated on the day of birth was assessed in rats of each genotype by quantifying the number of BrdU + cells still present at PN30 (Fig. 4). The number of rats included in analysis was: WT male + oil ($n = 7$), female + oil ($n = 7$), female + E2 ($n = 7$), female + DHT ($n = 7$), Tfm + oil ($n = 6$), Tfm + E2 ($n = 6$), Tfm + DHT ($n = 6$). In the dentate gyrus there was a significant effect of genotype ($F[2,39] = 59.65$, $p < 0.0001$, $\eta_p^2 = 0.78$), treatment ($F[2,39] = 52.759$, $p < .0001$, $\eta_p^2 = 0.73$) and a genotype $\times$ treatment interaction ($F[2,39] = 9.35$, $p < 0.0001$, $\eta_p^2 = 0.32$). Consistent with the higher rate of proliferation observed in WT males, they also had significantly more BrdUir cells at 30 days in the dentate gyrus than oil treated females ($p < 0.00001$, $d = 4.96$) and Tfm males ($p < 0.0001$, $d = 13.29$). Estradiol increased BrdUir cells significantly above oil treated female controls ($p < 0.0001$, $d = 5.49$) and the number of surviving cells in this group did not differ from WT males. Thus, administration of estradiol to females at birth increased the number of cells that survived to that of males. However, DHT did not promote survival in females as this group had the same mean number of BrdUir cells as females treated with oil, which was significantly less than that seen in WT males ($p < 0.00001$, $d = 3.97$). But, both estradiol and DHT increased the number of surviving BrdUir cells in the Tfm male dentate gyrus above that of oil treated Tfm males ($p < 0.0001$, $d = 3.17$ and $d = 4.28$ respectively), and the two hormone-treatment groups did not differ from one another. Nonetheless, Tfm males treated with DHT had significantly fewer surviving BrdUir cells than WT males ($p < 0.001$, $d = 4.04$).

Similar to the main effects in the dentate gyrus, analysis of BrdUir cells in CA1 found a significant effect of genotype ($F[2,39] = 40.32$, $p < 0.0001$, $\eta_p^2 = 0.67$) and hormone ($F[2,39] = 15.32$, $p < 0.0001$, $\eta_p^2 = 0.44$; Fig. 4), but the interaction was not significant. WT males retained more BrdUir cells at PN30 compared to oil treated females ($p = 0.0001$, $d = 3.33$) or Tfm males ($p < 0.0001$, $d = 5.74$). Estradiol treated females did not differ significantly from oil treated females, although it approached significance ($p < 0.07$), and estradiol treated females did not differ from WT males. Again DHT did not have a long term effect on cell survival in female rats. On the other hand, estradiol administration to Tfm males at birth increased the number of BrdU labeled cells in the DG at 30 days compared to their number in Tfm controls ($p < 0.039$, $d = 1.71$) but DHT did not ($p > .05$).

In CA3, there was again a significant effect of genotype ($F[2,39] = 69.37$, $p < 0.0001$, $\eta_p^2 = 0.78$) and treatment ($F[2,39] = 35.85$, $p < .0001$, $\eta_p^2 = 0.64$), but no interaction. Females treated with estradiol had increased surviving BrdUir cells compared to vehicle controls ($p < 0.0001$, $d = 3.80$) and this group was not different from oil treated WT males. WT male controls exhibited significantly more BrdUir cells than female controls ($p < 0.0001$, $d = 5.98$) or DHT treated females ($p < 0.0001$, $d = 4.43$). The number of BrdUir surviving cells also remained significantly higher in WT males than oil ($d = 7.30$), DHT ($d = 6.60$) or estradiol ($d = 2.16$) treated Tfm rats (all p values < 0.0001). Only estradiol significantly increased the number of BrdUir cells in the CA3 subregion at 30 days in Tfm males compared to oil treated Tfm controls ($p < 0.004$, $d = 1.87$).

Experiment 5: Impact of androgen insensitivity on generalization of contextual fear and extinction

Tfm and WT male rats were given context conditioning in one of two distinct contexts and tested for conditioned freezing in each context over two consecutive days. The first session is referred to as retention, and the second session is referred to as extinction. Repeated measures ANOVA with genotype as the between subjects variable and time as

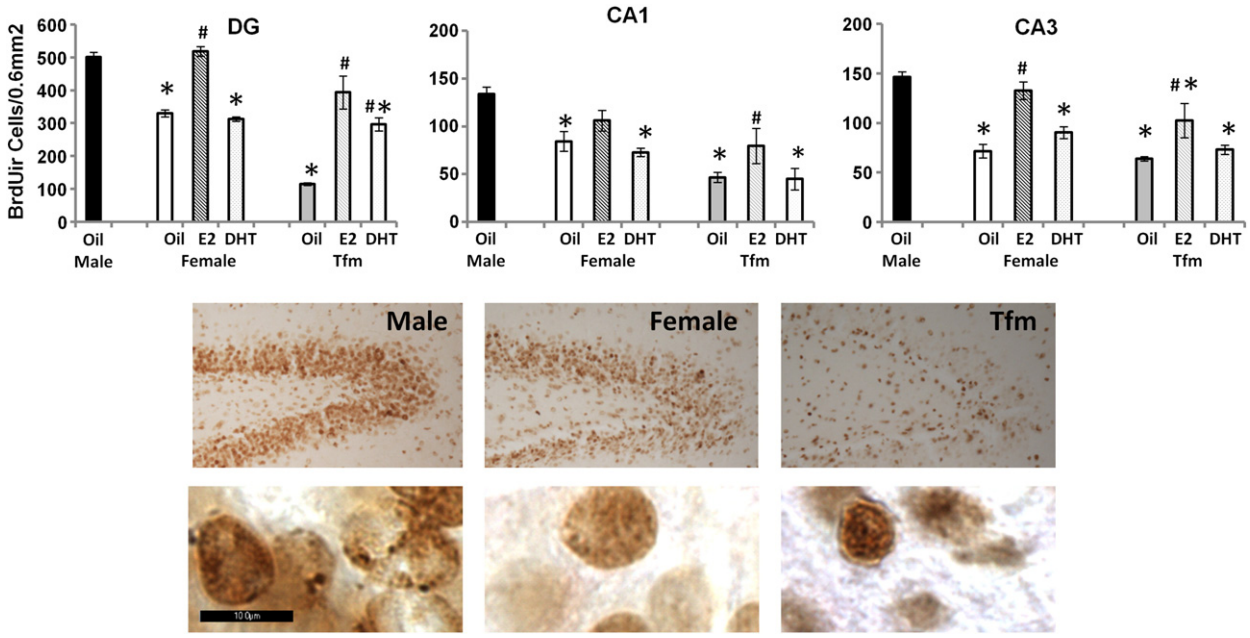

Fig. 4. Top panel: Wild type female and Tfm male pups were treated with oil vehicle, estradiol or DHT on the morning of birth. Wild type male pups were treated with oil vehicle. Two hours following steroid treatment, male, female and Tfm males were treated with a single dose of BrdU (100 mg/kg). All pups were euthanized 30 days following BrdU administration. * indicates significance difference from males; # indicates significant difference from same sex vehicle treated controls. Bottom panel: Representative images of BrdU immunoreactivity at PN30 in the dentate gyrus in a male, female and Tfm male at low magnification (top row, 20×) and high magnification (bottom row, 100×, scale bar = 10 μm). Cell survival was extremely reduced in Tfm males.

the within subjects variable was used to analyze suppression of movement prior to conditioning. Tfm males and WT male rats did not differ in activity levels in either context prior to fear conditioning. Thus, no pre-conditioning differences in locomotion were detected prior to administration of footshock. The same analysis of the post-training test of performance in the context paired with shock 24 h after conditioning did not find a significant effect of genotype, time, or a genotype × time interaction ($F[4,48] = 1.14$, $p > 0.05$). Tfm and WT male rats expressed equivalent levels of fear in the context paired with the footshock (Fig. 5). However, analysis of performance in the context not paired with shock revealed generalization of fear in the Tfm male rat. Analysis of performance during the retention test in the context never paired with shock did not find a significant effect of time or genotype × time interaction, but there was a significant effect of genotype ($F[1,12] = 5.72$, $p < 0.03$, $\eta_p^2 = 0.32$). Tfm rats displayed more fear in this context than WT males. Expression of fear persisted in both contexts for the second testing session in Tfm rats. Analysis of the extinction session in the shock context revealed a significant effect of genotype ($F[1,12] = 9.62$, $p < 0.009$, $\eta_p^2 = 0.44$). The effect of time was also significant ($F[4,48] = 2.93$, $p < 0.03$, $\eta_p^2 = 0.19$), whereas the genotype × time interaction was not ($F[4,48] = 1.12$, $p > 0.05$). Expression of fear persisted in the Tfm males, while extinction, or loss of fear was evident in WT male rats. The same was true in the no shock context. Freezing behavior in Tfm rats in the no shock context was persistent, demonstrating generalized fear. The main effect of genotype was significant ($F[1,12] = 9.822$, $p < 0.05$, $\eta_p^2 = 0.45$), indicating that the generalization of fear persisted longer in Tfm males, and that that fear appeared to be generally resistant to extinction. Repeated measures ANOVA did not detect a significant effect of time, or a time × genotype interaction.

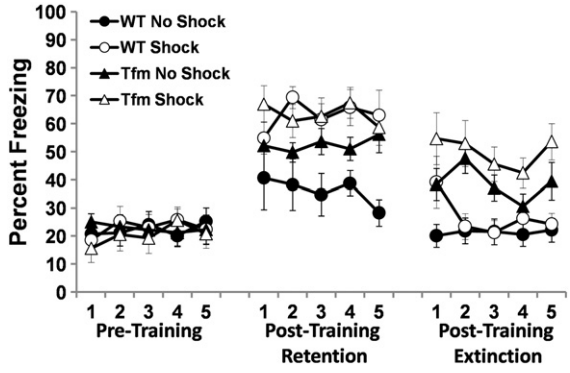

Fig. 5. Androgen insensitivity permits generalization of fear and resistance to extinction in the Tfm male. Wild type males and Tfm males were exposed to two distinct contexts. One context was paired with footshock. Tfm males spent significantly more time freezing in the context never paired with shock and freezing persisted longer in both context compared to wild type males. Data are presented in 2-min intervals.

Discussion

The Tfm rat is a unique tool to facilitate our understanding the role of androgen receptors in brain development. Our laboratory has established that both androgens and estrogens are involved in mediating the higher rate of cell genesis in the developing male hippocampus and that administration of both androgens and estrogens can increase cell genesis in the postnatal female hippocampus, but antagonists of the androgen receptor (i.e., flutamide) have produced unclear effects, acting as an antagonist in some instances but an agonist in others (MacLusky et al., 2004; Zhang et al., 2008). The Tfm male allowed us to extend our data to another rat strain (from Sprague Dawley to Long Evans) and further examine the developmental role of androgens in brain development. Cell genesis in the postnatal Tfm hippocampus was altered compared to unaffected males and females. The number of proliferating cells labeled with BrdU did not increase in number from 2 to 6 h on the day of birth in the Tfm hippocampus but did so in male and female rats in the dentate gyrus and CA1. This, coupled with significantly fewer Ki67ir cells, an additional marker of proliferating cells, in the Tfm male dentate gyrus and CA3 support the conclusion that progenitors in the Tfm

male hippocampus do not exit the cell cycle and survive at the same rate as in male and female rats.

The number of cells labeled at birth and quantified in the dentate gyrus at PN30 is lower in the Tfm male hippocampus compared to males or females. This is likely to have long term effects on hippocampal function, as cells generated at this time comprise the majority of cells in the dentate gyrus into adulthood (Muramatsu et al., 2007). It is possible that Tfm males do not exhibit normal cell genesis, survival or cell migration (e.g., Altman and Beyer, 1990). Administration of DHT or estradiol increased short term survival in the Tfm hippocampus. Supraphysiological doses of DHT can stimulate the Tfm rat androgen receptor (Langley et al., 1998; Yarbrough et al., 1990) and DHT administration increased the number of BrdUir cells in the Tfm male dentate gyrus at PN30. Cell numbers in the dentate gyrus of vehicle treated Tfm males was significantly less than vehicle treated females; DHT did not restore Tfm male cell number to that of wild type males, however. The absence of normal androgen binding in the Tfm male may impact sensitivity to endogenous estradiol. Estradiol, more consistently than DHT, enhanced cell genesis and/or survival in both the female and Tfm male hippocampus despite similar levels of endogenous estradiol (see also Konkle and McCarthy, 2011). Basal levels of cell genesis rely on estrogen binding in the neonatal male hippocampus; antagonism of estrogen receptors lowers cell genesis below that detected in female rat pups (Bowers et al., 2010). Further experiments are needed to determine whether loss of androgen signaling influences estrogen sensitivity in the developing hippocampus.

Insensitivity to endogenous estrogens and androgens might reduce GABA-induced excitation in the Tfm male hippocampus. Newly born neurons have higher intracellular chloride than mature neurons and thus respond to GABA-A receptor activation with a greater probability of depolarization and activation of L-type voltage-gated calcium channels (Ben-Ari, 2002; Obrietan and ven den Pol, 1995; Perrot-Sinal et al., 2003). Sex differences in chloride transporter expression correspond to robust GABA-mediated sex differences in measures of calcium-dependent cell excitability (Auger et al., 2001; Galanopoulou, 2008; Nunez and McCarthy, 2007; Perrot-Sinal et al., 2003). This sex difference is mediated by differential protein levels and activation of the cation-chloride cotransporter NKCC1, the cotransporter responsible for chloride accumulation inside the cell (Nunez and McCarthy, 2007; Nunez and McCarthy, 2008). Females "switch" to inhibitory GABA much earlier than males and thus GABAergic stimulation elicits calcium transients in male-derived hippocampal neurons for several days longer than in females (Nunez and McCarthy, 2007). Both androgens and estrogens modulate GABA-induced calcium transients in hippocampal neurons, but in differing ways (Galanopoulou, 2008; Nunez and McCarthy, 2007; Perrot-Sinal et al., 2003). Estradiol administration increases the number of cells responding to the GABA-A agonist muscimol with calcium transients as well as the magnitude of calcium influx (Nunez and McCarthy, 2007; Nunez et al., 2005). Androgens modulate the pattern of responding to repeated GABAergic stimulation in that female derived cells exhibit desensitization to GABA stimulation, and therefore do not respond to a second exposure to muscimol whereas male-derived cells, or female derived cells treated with DHT strongly respond to a second pulse of muscimol, suggesting androgens attenuate desensitization (Nunez and McCarthy, 2008). The GABAergic excitatory drive on newborn neurons is an important contributor to their survival and integration into the neural network (e.g., Ge et al., 2006; Pfeffer et al., 2009; Song et al., 2005; Tyzio et al., 1999). Adult hippocampal cell genesis relies on depolarizing GABA for proliferation, differentiation and incorporation into existing circuits (Deisseroth et al., 2004; Ge et al., 2006; Song et al., 2005; Tozuka et al., 2005). Loss of sensitivity to androgens may disrupt sex-specific modulation of depolarizing GABA and its influence on cell genesis. Whether adult neurogenesis in the adult Tfm is also dysregulated due to androgen insensitivity and possible changes in estradiol sensitivity remains to be determined.

Developmental insensitivity to androgens renders the Tfm male hyper-responsive to stress (e.g., Zuloaga et al., 2011a). The adult Tfm male exhibits an abnormally high and protracted elevation of corticosterone in response to mild stressors, such as placement in an open field, compared to WT males (Zuloaga et al., 2011a). The hippocampus serves as a source of negative feedback inhibition of the stress axis at the level of the hypothalamus, which regulates release of pituitary ACTH and corticosterone synthesis from the adrenal gland (for review see Jankord and Herman, 2008). The exaggerated stress response reported in adult Tfm males parallels that seen in animals with hippocampal lesions (Herman et al., 1998). Gonadectomy of adult male rats also increases circulating corticosterone levels, and permits higher and protracted elevations of ACTH following stress (Handa et al., 1994; Lund et al., 2004). Gonadectomy also results in activation of neurons in the periventricular nucleus (PVN) of the hypothalamus in adult male rats (Viau et al., 2003). These effects are reversed by testosterone or DHT, but not estradiol (Lund et al., 2004). Here we demonstrate that disruption of androgen signaling promotes generalization of contextual fear and resistance to extinction of this fear. This is likely due at least in part to the hypersecretion of corticosterone reported in these animals. Acquisition of contextual fear and other models of protracted fear resembling anxiety rely on corticosterone binding in the brain (Cordero et al., 1998; de Jongh et al., 2002, 2003; Waddell et al., 2008; Walker et al., 2009). Little is known about how gonadal steroids at birth modulate future expression of fear and anxiety once acquired. Though estradiol cannot reverse many changes in the stress axis elicited by gonadectomy in adulthood, developmental insensitivity to androgens as is the case in the Tfm male may influence sensitivity to or production of estrogen metabolites. Androgen binding stimulates aromatase activity, and thus conversion of testosterone to estradiol (Roselli and Klosterman, 1998). The androgen receptor mutation might similarly change conversion of DHT to 3β-diol, or sensitivity of the ERβ receptor, disrupting the effects of estrogens at ERβ receptors (Pak et al., 2005). ERβ mediates many of the known effects of androgens on the stress axis (Handa et al., 2008; Lund et al., 2005, 2006).

Gonadal steroids at birth organize sex specific learning strategies and stress responses that rely on the hippocampus. Administration of testosterone to neonatal female rats masculinizes response strategy in the hippocampal dependent spatial water maze task and pyramidal cell morphology (Isgor and Sengelaub, 2003). Early testosterone similarly masculinizes a sexually dimorphic response to uncontrollable stress, as well as stress induced spinogenesis in the hippocampus (Bangasser and Shors, 2007; Shors, 2001; Shors and Miesegaes, 2002; Wood and Shors, 1998). We did not test females in the fear conditioning experiment presented here because female rats spend little time freezing to conditioned contextual cues compared to males, as freezing is interrupted by exploratory behaviors in females (Anagnostaras et al., 1994; Barker and Galea, 2010). We confirmed that Tfm rats do not exhibit a female-like response during contextual fear conditioning, suggesting that Tfm males express fear through a freezing conditioned response as do normal males, but that fear is robustly exacerbated. Insensitivity to or removal of androgens has a disinhibitory effect on the stress axis (Handa et al., 1994; Viau and Meaney, 1996; Zuloaga et al., 2011a, 2011b). Whether the sex differences and hormonal modulation of neonatal cell genesis that we observe are functionally related to the differences in contextual fear conditioning between wild type males and Tfm males is unknown. Altered neurogenesis in early development might disrupt the establishment of anatomical connections between the hippocampus and other brain regions involved in stress responding and generalization of fear, such as the amygdala (DonCarlos et al., 2003; Sarkey et al., 2008). Androgen receptor immunoreactivity has been demonstrated on axons and dendrites innervating the amygdala (DonCarlos et al., 2003). Alternatively, loss of androgen sensitivity at nuclear androgen receptors may dysregulate the normal balance of corticotrophin releasing factor (CRF) receptors CRF1 and CRF2. Application of DHT increases CRF2 receptor mRNA in hippocampal cell cultures (Weiser et al., 2008) and CRF2 receptor knockout mice display exacerbated anxiety and sensitivity to stress (Bale et al., 2000). Altered neonatal cell genesis during a developmental sensitive period of androgen

action may ultimately lead to altered hippocampal stress responding in adulthood as we and others have seen in adult Tfm male rats. Thus, the Tfm model in which genetic males have dysfunctional ARs is likely to continue to offer new insights into the organizational and developmental influence of testosterone and its metabolites on the stress axis.

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