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Neonatal testosterone partially organizes sex differences in stress-induced emotionality in mice

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Abstract

Major depressive disorder (MDD) is a debilitating disorder of altered mood regulation. Despite well established sex differences in MDD prevalence, the mechanism underlying the increased female vulnerability remains unknown. Although evidence suggests an influence of adult circulating hormone levels on mood (i.e. activational effects of hormones), MDD prevalence is consistently higher in women across life stages (and therefore hormonal states), suggesting that additional underlying structural or biological differences place women at higher risk. Studies in human subjects and in rodent models suggest a developmental origin for mood disorders, and interestingly, a developmental process also establishes sex differences in the brain. Hence, based on these parallel developmental trajectories, we hypothesized that a proportion of the female higher vulnerability to MDD may originate from the differential organization of mood regulatory neural networks early in life (i.e. organizational effects of hormones). To test this hypothesis in a rodent system, we took advantage of a well-established technique used in the field of sexual differentiation (neonatal injection with testosterone) to masculinize sexually dimorphic brain regions in female mice. We then investigated adult behavioral consequences relating to emotionality by comparing neonatal testosterone-treated females to normal males and females. Under baseline/trait conditions, neonatal testosterone treatment of female mice did not influence adult emotionality, but masculinized adult locomotor activity, as revealed by the activational actions of hormones. Conversely, the increased vulnerability of female mice to develop high emotionality following unpredictable chronic mild stress (UCMS) was partially masculinized by neonatal testosterone exposure, with no effect on post-UCMS locomotion. The elevated female UCMS-induced vulnerability did not differ between adult hormone treated groups. These results demonstrate that sex differences in adult emotionality in mice are partially caused by the organizational effects of sex hormones during development, hence supporting a developmental hypothesis of the human adult female prevalence of MDD.

Keywords

depression; emotionality; testosterone; unpredictable chronic mild stress; sex difference; development

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INTRODUCTION

Major depressive disorder (MDD) is a devastating chronic illness of altered mood regulation affecting approximately 151 million people worldwide. Importantly, patients with mood disorders (i.e. MDD or bipolar disorder) account for approximately 60% of completed suicides (Mann, 2003), highlighting the profound consequences of the disease. In addition to the psychological stress on patients and families, MDD contributes to the development and progression of systemic and organ diseases (Ciechanowski et al., 2000; Musselman et al., 1998; Schulz et al., 2000), extending the health impact of MDD beyond the realm of psychiatry. Women are approximately twice as likely to experience a single MDD episode as men (Kornstein et al., 2000) and four times as likely to have recurrent MDD (Perugi et al., 1990). Women also have more MDD symptoms and greater symptom severity (Angst and Dobler-Mikola, 1984; Frank et al., 1988; Young et al., 1990). The marked sex difference in MDD incidence is often attributed to the greater likelihood of women to seek treatment for the disorder; however, differences are also observed in community-based epidemiological studies, where the factor of seeking treatment is removed (Angst and Dobler-Mikola, 1984), suggesting underlying biological predisposing factors in female subjects. If these sex differences in MDD incidence, symptomatology, and/or severity are due to underlying biological sex differences, a more thorough understanding of the biology is warranted to develop better treatment or even prevention of MDD.

Numerous human studies have identified early life experience as a mediating factor in the development of adult mood, with early life trauma, abuse, and neglect increasing risk for adult mood and anxiety disorders (reviewed in Heim and Nemeroff, 2001, and Pine and Cohen, 2002). The developmental origin of adult emotionality (defined as measurable behavioral and physiological components of emotions or of their equivalents in rodents) has also been extensively studied in rodents, where early life maternal care, stress, and/or genetics can be manipulated, leading to changes in adult emotionality (reviewed in Leonardo and Hen, 2008; Pryce et al., 2005).

Hormonally-influenced sex differences are caused by either activational or organizational effects of hormones (reviewed in Cooke et al., 1998). Briefly, circulating gonadal hormones can have acute and transient effects that can occur throughout life (activational), while exposure to gonadal hormones during critical periods of development can cause permanent sex differences (organizational). A number of studies suggest links between adult female hormone levels and mood states (activational effects), particularly in relation to premenstrual dysphoric disorder (PMDD) and postpartum depression (reviewed in Joffe and Cohen, 1998; Moses-Kolko et al., 2009; Rubinow et al., 1998). Many animal studies have also revealed a link between circulating hormone levels and emotionality (e.g. Goel and Bale, 2008; Viau and Meaney, 1991). However, MDD prevalence is higher across life stages (Castro-Costa et al., 2008; Kessler et al., 1993), and therefore hormonal states, suggesting underlying structural or biological differences that place women at higher risk.

The organizational actions of gonadal hormones are known to cause a number of sex differences in behavior and brain structure, although a majority of the literature concerning organizational sex differences involve sex behavior and brain regions controlling sex behavior in rodents (Handa et al., 1985; Phoenix et al., 1959). An extensively used and validated method to masculinize the brain and behavior in female rodents is neonatal testosterone treatment, where a single dose of testosterone on the day of birth consistently induces adult male sexual behavior and brain anatomical patterns in female mice and rats (Guillamon et al., 1988; Hisasue et al., 2010; Mong et al., 1999; Murray et al., 2009) (reviewed in Arnold and Gorski, 1984; Morris et al., 2004; Negri-Cesi et al., 2004; Simerly,

2002). Hence, in view of the developmental contribution to adult mood regulation, we hypothesized that sex differences in the vulnerability to develop episodes of MDD are, at least partially, organized by sex hormones early in life. While some studies have investigated organizational sex differences in emotionality in rodents, most of these studies have examined animals under baseline conditions, in which sex differences in emotionality are modest and often undetected (Goel and Bale, 2008; Zuloaga et al., 2011a; Zuloaga et al., 2011b). To model in rodents the observed human sex difference in vulnerability to develop MDD, it is critical to induce a depressive-like state using a paradigm with observed sex differences favoring female vulnerability. Accordingly, we recently showed that female mice are more susceptible to developing a depressive-like syndrome following unpredictable chronic mild stress (UCMS) (Guilloux et al., 2011). Hence, in this study, we quantified emotionality under both baseline (trait-like) and UCMS-induced states in normal males, normal females, and females treated neonatally with testosterone, while controlling for potential activational effects of estradiol (E₂) by comparing behavior in control mice to gonadectomized (GDX) and E₂-supplemented mice.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6NTac breeding pairs of mice (Taconic; Hudson, NY) were maintained under standard conditions (12/12 hour light/dark cycle, 22 ± 1°C, food and water ad libitum), in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

Experimental Design

Refer to Figure 1A for the time course of the experimental design and Table 1 for group names as well as N's. Mice were injected with either testosterone propionate (TP) or peanut oil (vehicle) on the day of birth (postnatal day 0 (PO)) and then left undisturbed and un-manipulated until adulthood. At approximately 5 months of age, half the mice from each neonatal treatment group were GDX and implanted with an E₂ capsule, while the other half of each neonatal treatment group was given sham surgery and blank implant. E₂ replacement was used since, in un-manipulated males, testosterone is converted to E₂ by aromatase. Thus, females are normally exposed to E₂ secreted from the ovaries and males are normally exposed to E₂ that is converted from testosterone secreted from the testes. After allowing one month of recovery from surgery and for hormone levels to equilibrate, baseline behavior was assessed using the elevated plus maze (EPM), open field (OF) and novelty suppressed feeding (NSF) tests. The tests were performed 3–5 days apart. Mice were then exposed to 8 weeks of UCMS. During weeks 7 and 8 of UCMS, animals were exposed to the same panel of behavioral tests, with the addition of the forced swim test (FST), to establish post-UCMS emotionality and locomotion. FST was not performed at baseline due to the strong stressor component of this test. 6 months of age correspond to an adult period where we have previously reported potent effects of UCMS on emotionality (Edgar et al., 2011). A longitudinal study (i.e. baseline behavior followed by UCMS exposure followed by post-UCMS behavior) was employed due to experimental constraints on the number of animals that can be used in a UCMS/behavior study, since the focus of the experimental contrasts were on group differences under baseline and high emotionality states.

Hormone manipulation and assay

Females were injected subcutaneously with 100µg of TP in 25µL peanut oil (Hisasue et al., 2010), or an equal volume of oil on the day of birth; males were injected with oil. Females injected neonatally with testosterone are referred to as “NeoM Females”; females injected

neonatally with oil are “Normal Females”; males injected neonatally with oil are “Normal Males”. Note that “Normal” refers to normal developmental hormone exposure.

Under anesthesia, adult mice were bilaterally GDX to remove endogenous, gonadal sources of sex hormones, or sham surgery was performed. At the time of surgery, GDX animals received a subcutaneous SILASTIC (Dow Corning Corp., Midland, MI) capsule containing a 1:1 mixture of 17β -E₂ and cholesterol (1.02mm I.D./2.16mm O.D.; 5mm length; as in (Bodo and Rissman, 2008)) and sham animals received blank capsules. Due to required length of time to complete baseline behavior and UCMS (~10 weeks), we were concerned that the capsules would become “walled-off” by connective tissue, preventing hormone release. Accordingly, we implanted fresh (2mm length) hormone-filled or blank capsules on the first day of UCMS. At the time of sacrifice, we collected trunk blood for E₂ assay. Whole blood samples were allowed to clot at room temperature for 90 minutes, followed by centrifugation to isolate serum. Serum samples were sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (supported by the Eunice Kennedy Shriver NICHD/NIH (SCCPIR) Grant U54-HD28934) and ELISA (Calbiotech; Spring Valley, CA) was used to determine E₂ concentration.

Unpredictable chronic mild stress (UCMS)

UCMS replicates the role of stress in eliciting MDD, models several MDD symptom dimensions, and respects the timeframe of onset and efficacy of antidepressant treatment (Surget et al., 2009). Refer to Supplementary Table 1 for details on the UCMS schedule of stressors. Briefly, the UCMS protocol consisted of an 8-week period during which group-housed mice were exposed to a randomized schedule of environmental disturbances approximately 1–2 times per day, seven days a week, as applied in our lab (Edgar et al., 2011; Joeyen-Waldorf et al., 2009; Surget et al., 2009). Disturbances included forced bath (~2cm of water for 15min), aversive smell (1 hour exposure to fox urine), light cycle reversal or disruption, social stress (rotate mice into previously occupied cages), tilted cage (45° tilt), mild restraint (50ml conical tube with air hole for 15 min), bedding change (replace soiled bedding with clean bedding), wet bedding, and no bedding.

Behavioral testing

Elevated plus maze (EPM)—Behavior in the EPM was measured as previously described (Sibille et al., 2000) using a cross maze with 2 open and 2 closed 30 × 5 cm arms. Time spent and percent entries (entries into open arms divided by entries into open or closed arm × 100) in the open arms were recorded for 10 min to measure anxiety-like behavior. The total number of entries into any arm was used as an index of locomotor behavior.

Open field (OF)—The OF test was performed in a photo beam tracking arena (25.4 × 25.4 cm; TruScan; Coulbourn Instruments, Allentown, PA). The center of the OF was defined as the centermost 18 × 18 cm of the arena. The time spent and percent distance (distance in center divided by total distance × 100) in the center of the arena were recorded for 10 minutes as measures of anxiety-like behavior. The total distance travelled was recorded as an index of locomotor activity.

Novelty suppressive feeding (NSF)—The latency for food-deprived mice to feed in an aversive, novel environment was used as an index of emotionality (Edgar et al., 2011). Mice were food-deprived for 16 hours prior to exposure to NSF. Testing was performed in a brightly lit 51 × 51 cm arena covered in bedding. Latency to eat a food pellet placed in the aversive center of the arena was recorded during a 12 minute session. Food consumption in the home cage (food eaten divided by body weight) during 8 minutes following NSF testing

and percent weight lost during food deprivation were measured as controls for appetite differences.

Forced swim test (FST)—Mice were assessed for acute stress response in the FST. Briefly, mice were placed in a 1 liter beaker filled with room temperature (~25°C) water for 6 minutes. During the last 4 minutes of testing, time swimming was measured as index of stress-responsiveness. Here, increased swimming duration was interpreted as positive coping behavior when faced with an acute, life-threatening environmental challenge. Due to its high stress component, the FST was only performed following UCMS.

Emotionality and locomotion Z-scores

When multiple behavioral tests are performed over a period of several days, measures of dependent variables tend to be sensitive to multiple known (and unknown) environmental factors (time of day, animal facility-related, experimenter, etc). While we report results of individual tests, we have also developed a complementary procedure of Z-score normalization of data, based on clinical meta-analysis Z-score principles, to obtain an overall “emotionality score” and “locomotion score” for each animal. See details and rationale in (Guilloux et al., 2011). In short, Z-scores are standardized to the mean and standard deviation of a comparison group and no normal assumption is made. These scores indicate how many standard deviations (σ) an observation (X) is above or below the mean of the comparison group (μ).

$$z = \frac{X - \mu}{\sigma}$$

X represents the individual data for the observed parameter. μ and σ represent the mean and the standard deviation for the control group, respectively. These scores integrate emotionality-related behaviors first within tests, then across tests, and provide a reliable, meaningful picture of the overall emotional and locomotor profile of animals over time, potentially similar to protocols for measuring mood states in humans, as a set of symptoms consistently expressed, but whose nature varies greatly over time. This approach is different from a principal component analysis (PCA), which assumes that consistent behaviors are systematically observed across tests and time. Also, contrary to PCA analysis, Z-scores provide clear, summarized quantitative values that are highly useful for direct statistical analysis of group differences. Briefly, data is collected from multiple validated behavioral tests, including NSF (1 emotionality measure), EPM (2 emotionality and 1 locomotor measures), OF (2 emotionality and 1 locomotor measures), and FST (1 emotionality measure). For each behavioral test measure, an individual’s score is Z-normalized to the mean and standard deviation of a control group (here, Normal Female was the comparison group). For EPM and OF, in which two emotionality measures are scored, the test specific Z-scores are averaged within behavioral tests. All behavior tests are then equally weighted in combined average Z-scores to obtain separate overall emotionality and locomotor scores for each animal.

Estrous cycle testing

Phase of the estrous cycle was determined in female mice by vaginal smears following each behavioral test (Caligioni, 2009). Briefly, approximately 10 μ l of sterile saline was flushed into the vagina and placed onto a slide. After coverslipping, the slides were examined under a light microscope with 10 $\times$ objective to determine phase of estrous cycle. Male groups experienced anogenital stimulation with a pipette tip.

Anatomical measurement

To verify the effect of neonatal treatment on masculinization of the brain (Hisasue et al., 2010), we measured volume of the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) in a separate cohort of mice receiving the exact neonatal treatment described above (males injected with oil; females injected with oil or TP). In adulthood, mice were sacrificed by avertin overdose and brains were post-fixed in 5% acrolein for 4 hours and stored in 30% sucrose until sectioning. Brains were sectioned on a cryostat at 40µm, mounted onto gelatin-subbed slides, and stained with thionin. BNSTp volume was determined by bilaterally tracing BNSTp outline in alternate sections throughout the rostral-caudal extent of the nucleus using MetaMorph software (Molecular Devices, Sunnyvale, CA). Volume was calculated by multiplying the summed area by the sampling ratio (2) and section thickness. Slides were coded so that the experimenter was blind to treatment group.

Statistical Analysis

We used two-way ANOVA (neonatal hormone-by-adult hormone) to compare groups for each behavioral test dependent measure. Only if the two-way ANOVA was significant for main effect of neonatal hormone, main effect of adult hormone, or interaction of neonatal and adult hormone did we perform planned contrasts using unpaired two-tailed t-tests. If there was a significant main effect of neonatal hormone, sex differences were tested by comparing Normal Males to Normal Females and the organizational hormonal drive was tested by comparing NeoM Females with both Normal Females and Normal Males. If there was a significant neonatal-by-adult hormone interaction, we contrasted Shams and E-Clamps within each neonatal treatment to test for activational effects of hormones (Summarized in Figure 1B). To determine whether any dependent variable was influenced by estrous state, we analyzed dependent measures with one-way ANOVA (by estrous phase). For the E₂ assay, one-way ANOVA was used to compare all six groups (Normal Female/Sham, Normal Female/E-Clamp, Normal Male/Sham, Normal Male/E-Clamp, NeoM Female Sham, NeoM Female/E-Clamp), followed by planned comparisons. BNSTp volume was analyzed by one-way ANOVA (by neonatal treatment group), followed by planned comparisons. All data are expressed as mean ± SEM and statistical significance was set at $p < 0.05$. Trend level was set at $0.05 < p < 0.1$.

RESULTS

Biological verification of hormone treatments

Neonatal TP and BNSTp volume—We observed a main effect of neonatal treatment on BNSTp volume ($p < 0.001$; Figure 2A). Posthoc analysis indicated that Normal males had significantly larger BNSTp volume than Normal Females ($p < 0.001$). NeoM Females displayed intermediate BNSTp volumes compared to Normal Females and Normal Males ($p < 0.1$ for both comparisons).

GDX and adult E₂ replacement levels—A main effect of group on serum E₂ levels was observed ($p < 0.001$; Figure 2B). As expected, E₂ levels were significantly elevated in GDX/E₂-clamped groups compared to matched shams ($p < 0.0025$ for all comparisons) and did not differ across E-Clamp groups ($p > 0.1$ for all comparisons). There were no differences in E₂ levels among the sham groups ($p > 0.8$ for all comparisons).

Neonatal testosterone treatment alters adult locomotion, but not emotionality, under baseline trait-like conditions

Emotionality—Refer to Table 2 for a summary of baseline emotionality-related behavior statistics. In the EPM (Figure 3A-B), there was no main organizational effect, no main

activational effect, and no interaction of neonatal and adult hormones on time spent in the open arms. For percent crosses into the open arms of the EPM, there was no main organizational effect and no neonatal-by-adult hormone interaction, but there was a trend for activational effects, with Shams having higher percent crosses into the open arms than E-Clamps (i.e. lower emotionality in Shams).

In the OF (Figure 3C-D), there was no main organizational effect and no neonatal-by-adult hormone interaction on time in the center of the chamber; there was a trend for Shams to spend more time in the center than E-Clamps (i.e. lower emotionality in Shams). For percent distance in the center of the OF, there was no main activational effect and no neonatal-by-adult hormone interaction. A main organizational effect was observed on percent distance in the center of the OF; planned contrasts revealed that Normal Males had higher percent distance center (lower emotionality) than both Normal Females and NeoM Females. In the NSF (Figure 3E), there was no main organizational effect and no neonatal-by-adult hormone interaction on latency to eat. A main activational effect was observed on latency to eat in the NSF (i.e. reduced latency in E-Clamps). Notably, there was also a main activational effect on homecage food consumption following NSF (E-Clamps ate more than Shams), and main activational and organizational effects on weight lost during overnight food deprivation (Shams lost less than E-Clamps; Normal Males lost less than Normal Females, which in turn lost less than NeoM Females); there was no neonatal-by-adult hormone interaction for either test (Table S2). So the main activational effect on latency in NSF (“adult hormones” in Figure 3E) could be explained by group differences in appetite rather than emotionality.

None of the behavioral emotionality measures in the EPM, OF, and NSF varied by phase of the estrous cycle ($p > 0.4$ for all measures). The combined Z-score (Figure 3F) confirmed the overall lack of baseline emotionality differences (no organizational or activational effect; no neonatal and adult hormones interaction).

Locomotion—Refer to Table 2 for a summary of baseline locomotor behavior statistics. In the EPM (Figure 4A), we detected a main organizational effect on total crosses in the EPM; planned contrasts revealed that Normal Females had greater number of total crosses than both NeoM Females and Normal Males. There was no main activational effect and no neonatal-by-adult hormone interaction on total crosses.

In the OF (Figure 4B), we detected a significant main organizational effect. Planned contrasts revealed that Normal Males had shorter total distance than both Normal Females and NeoM Females. We also detected a significant neonatal-by-adult hormone interaction, with NeoM/E-Clamp animals having shorter total distance than NeoM/Shams. There was no main activational effect. The behavioral locomotion measures in the EPM and OF did not vary by phase of the estrous cycle ($p > 0.4$ for all measures).

The analysis of the combined baseline locomotion Z-scores confirmed the presence of a significant main organizational effect, where Normal Males exhibited lower locomotion than Normal Females and NeoM Females (Figure 4C). We also detected a significant neonatal-by-adult hormone interaction, with NeoM/E-Clamp animals having lower locomotion than NeoM/Shams. There was no activational effect in Normal Females and Normal Males.

Neonatal testosterone treatment partially masculinizes the UCMS-induced vulnerability of female mice to develop high emotionality states

Emotionality—Refer to Table 3 for a summary of post-UCMS emotionality-related behavior statistics. In the EPM (Figure 5A-B), there were significant main organizational effects on both the time in the open arms and on the percent crosses into the open arms. Planned contrasts revealed that Normal Males spent more time in the open arms and had

higher percent crosses into the open arms (lower emotionality) than both Normal Females and NeoM Females. There were no main activational effects and no neonatal-by-adult hormone interaction.

In the OF (Figure 5C-D), there was no main organizational effect and no neonatal-by-adult hormone interaction on time spent in the center or on percent distance in the center, but we detected a main activational effect on these measures (i.e. lower emotionality in Shams). In the NSF (Figure 5E), there was no main organizational effect and no neonatal-by-adult hormone interaction on latency to eat. There was a significant main activational effect on latency, with E-Clamps eating faster than Shams (i.e. lower emotionality in E-Clamps). Following NSF, we observed main activational and organizational effects on homecage food consumption (E-Clamps ate more than Shams; NeoM Females ate more than Normal Males and Females; $p < 0.05$ for all measures) and on weight lost during overnight food deprivation (Shams lost less than E-Clamps; Normal Males lost less than Normal Females and NeoM Females; $p < 0.05$ for all measures); there was no neonatal-by-adult hormone interaction for either test (Table S1). Thus, similar to baseline measures, the main activational effect on latency in NSF could be explained by group differences in appetite. In the FST (Figure 5F), there was no main organizational effect and no neonatal-by-adult hormone interaction on time swimming. There was a significant main activational effect, with Shams swimming more than E-Clamps (i.e. lower emotionality in Shams). None of the behavioral measures in EPM, OF, and NSF varied by phase of the estrous cycle ($p > 0.3$ for all measures).

Although driven by the EPM results, a significant main organizational effect on post-UCMS emotionality was maintained when combining emotionality measures across tests (Z-score, Figure 5G), where Normal Females displayed the highest emotionality scores compared to both NeoM Females and Normal Males. NeoM Females had a trend for higher emotionality scores than Normal Males, together suggesting an intermediate emotionality phenotype in NeoM females. The inconsistency of the activational effect in individual tests (increased emotionality in OF and FST, but decreased in NSF) resulted in no significant combined effects (z-scores). There was also no interaction of neonatal and adult hormones on combined scores.

Locomotion—Refer to Table 3 for a summary of post-UCMS locomotor behavior statistics. For locomotion measures (Figure 6), there was no organizational effect, no activational effect, and no neonatal-by-adult hormone interaction in the EPM, OF, and combined Z-scores. Locomotion measures did not vary by phase of the estrous cycle ($p > 0.1$ for all measures).

DISCUSSION

Focusing on the potential developmental role of hormones in establishing adult sex differences in emotionality, we tested the impact of neonatal testosterone exposure – a validated approach to developmentally "masculinize" the brain –, on adult anxiety- and depressive-like behaviors in mice. Here we show that neonatal testosterone exposure did not affect baseline (non-stress) results, but partially masculinized the UCMS-induced higher emotionality of female mice; NeoM females displayed emotionality measures that were intermediate between normal males and females (Figure 5G). These behavioral results are consistent with our observation of intermediate masculinization of BNSTp volume (Figure 2), a control test for neonatal TP treatment effectiveness. Our results also suggest that baseline sex differences in locomotor activity are established by hormonal exposure during development, although this was only revealed by the activational effect of E_2 hormones in NeoM Females (Figure 4C). The UCMS intervention eliminated sex differences in

locomotion (Figure 6). Overall, we did not observe consistent activational effects of E₂, although these studies were not designed to maximize these contrasts. Together, these results indicate that sex differences in adult emotionality are partially established by the organizational effects of sex hormones during development, hence providing evidential support from a mouse model for the hypothesis that sexual dimorphic development may contribute to the adult female prevalence of MDD.

Neonatal programming of induced state, but not baseline/trait emotionality

The fact that we did not observe sex differences in emotionality under baseline non-stressed conditions was not unexpected. These sex differences are often subtle, requiring either very large groups of mice or the combination of data from many studies to detect (as discussed in Guilloux et al., 2011). Some studies even report opposite directions of sex difference (i.e. males or females exhibiting higher emotionality) for baseline emotionality measures, depending on the behavioral test or genetic strain of mice used (reviewed in Palanza, 2001). For instance, when examining several commonly used strains of mice, (Voikar et al., 2001) found varying levels and directions of sex differences across multiple tests for anxiety- and depressive-like behavior under baseline, non-stressed conditions. In particular, C57BL/6 (used in this study) showed a lack of baseline sex difference on multiple measures across multiple behavioral tests (Voikar et al., 2001). One could argue that a different mouse strain may be more appropriate when studying baseline emotionality, but we used C57BL/6 mice in the current study due to their increased female susceptibility to develop an UCMS-induced depressive-like syndrome (Guilloux et al., 2011), which we speculate may better model the increased human female vulnerability to develop MDD episodes. It is possible that we would have seen a sex difference in baseline emotionality had the number of animals in each group been larger.

Our results suggest that neonatal testosterone exposure only partially masculinized post-UCMS emotionality, with NeoM Females exhibiting significant differences from Normal Females and only trend level differences from Normal Males. So, while statistically similar, a close inspection of the data suggests that NeoM females may in fact display a phenotype that is intermediate between Normal Females and Normal Males. Looking at the individual behavior tests that contribute to the post-UCMS emotionality Z-score (Figure 5), NeoM Females did not behave as Normal Males. For instance, in the EPM, Normal Males spent significantly more time and had higher percent crosses into the open arms than NeoM Females (i.e. Normal Males exhibited lower anxiety-like behavior). Although not significant, NeoM Females tended to show lower anxiety-/depressive-like behavior in the NSF and FST. The absence of effect in the OF test could reflect test specificities or variability in emotionality measures over days. Here, we speculate that it also may depend on the apparatus, as that specific automated system ended up being less sensitive to anxiety-like measures than previously-used systems (based on several experiments run over time), due to a smaller open area. Despite these potential limitations, the results from the combined Z-score analysis all together provided the expected significant male-female differences, and an intermediate phenotype in NeoM Females. Interestingly, this intermediate behavioral phenotype matches our results for BNSTp volume (Figure 2). Although other studies have reported complete masculinization of BNSTp volume by neonatal TP exposure in mice (Hisasue et al., 2010; Murray et al., 2009), our intermediate results may reflect differences in laboratory, procedures, and/or genetic background (C57Bl/6 versus C57Bl/6NTac here).

Activational effects of E₂ on emotionality

Although some activational effects of E₂ were observed for specific behavioral measures, these results were inconsistent across tests. For instance, GDX mice given E₂ capsules exhibited higher emotionality in the OF and FST than shams, but lower emotionality than

shams in the NSF. These inconsistent results cancel each other out and result in no overall activational effect in the emotionality Z-score. Previous studies report both anxiolytic and anxiogenic effects of E₂ on behavior in rodents, often dependent upon the dose and duration of treatment or on the specific test used (Mora et al., 1996; Morgan and Pfaff, 2001; Nomikos and Spyraiki, 1988). Consistent with our OF results, Morgan and Pfaff (2001) found that female mice that were GDX and given E₂ treatment (resulting in similar blood estrogen levels as here) spend less time in the center of the OF than mice treated with vehicle. Evidence suggests that the dichotomous actions of on emotionality may be due to its actions at either estrogen receptor alpha (ER α) or beta (ER β). For instance, treatment of GDX female rats with an ER α -specific agonist increases anxiety-like behavior, while treatment with an ER β -specific agonist decreases anxiety-like behaviour (Lund et al., 2005). Thus, our inconsistent results of E₂ treatment on emotionality may be due to actions via either ER α or ER β .

Other factors that could influence adult emotionality

If the sex difference in UCMS-induced emotionality is only partially due to organizational actions of testosterone on the day of birth (and adult circulating hormones do not influence this measure, as shown in our results), what other mechanisms could contribute to residual sex differences? It is possible that our developmental testosterone exposure either partially missed the critical period for masculinization (i.e. the critical period begins during late prenatal development (Simerly et al., 1985) or extends into the first few days after birth), or was too low of a dose. However, a similar neonatal dose of testosterone is sufficient to masculinize other sexually dimorphic measures in rodents (Breedlove et al., 1982; Hisasue et al., 2010; Jacobson et al., 1981; Murray et al., 2009; Murray et al., 2011). Differences in genetic sex could also play a role in emotionality; even though our neonatal testosterone treatment to females aimed to masculinize neonatal hormone exposure, the testosterone treated females were still genetically female for their entire lives, hence distinct from Normal Males. Therefore, genetic sex, regardless of developmental or adult hormone exposure, could represent an additional factor contributing to adult emotionality. Genetic males have only one X chromosome and one Y chromosome, while genetic females have two X chromosomes, and genes on the Y chromosome or gene dosage of the X chromosome could play a role in sex differences (McCarthy and Arnold, 2011). Since it is impossible to separate the potential role of genetic sex from gonadal (and therefore, hormonal sex) in traditional wild-type mice (as used here), genetic manipulation has been used to engineer mice in which genetic and gonadal sex can be dissociated, i.e. the Four Core Genotype (FCG) mice (reviewed in Arnold and Chen, 2009). The FCG mice provide the opportunity to specifically test for genetic sex differences, while keeping gonadal sex the same (and vice versa). Using the FCG mice, studies have shown that sex chromosome complement influences sexual differentiation of many neuronal phenotypes and behaviors, including vasopressin innervation in the lateral septum (De Vries et al., 2002), aggressive and parental behavior (Gatewood et al., 2006), and social behavior (Cox and Rissman, 2011; McPhie-Lalmansingh et al., 2008). We are currently using the FCG mice to further elucidate the potential contributions of gonadal sex and genetic sex to baseline and stress-induced emotionality.

It is also possible that post-UCMS emotionality was not completely masculinized in NeoM Females due to peripubertal hormone levels. There is evidence that puberty is another organizational period of sexual differentiation of the brain and behavior (reviewed in Schulz et al., 2009)). For instance, testosterone exposure during puberty masculinizes adult reproductive behavior in guinea pigs (Schulz et al., 2004). Since our neonatal testosterone treated females were hormonally distinct from Normal Males during the pubertal period,

these hormone differences could have differentially organized brain regions involved in emotionality.

Sex differences in locomotion

In the current studies, we observed a baseline sex differences in locomotion, with Normal Males having lower locomotor activity than Normal Females. This sex difference in locomotion was expected, as many studies report that female mice have higher locomotion levels than males under baseline stress conditions (Archer, 1975). Interestingly, we report the presence of an organizational drive on baseline locomotion that was only revealed under chronic E₂ conditions in NeoM Females (Figure 4C). The idea that hormones can activate tissues that are otherwise organized during development is not novel, as it has been consistently shown with respect to sex behavior. For instance, (Phoenix et al., 1959) found that the organizing action of testosterone on male sex behavior is only revealed after activation by adult testosterone. After UCMS, there was no sex difference in locomotor activity, indicating that chronic stress dampens the locomotor sex difference. It is important to note that the locomotion measures used here may still include an emotionality component, as they were measured when mice were subjected to a novel environment (i.e. during the 10 minutes of EPM and OF testing). In future studies, it will be interesting to assess locomotor activity during a longer timeframe (e.g. 2 hours in a locomotor box) and/or in the home cage, where response to a novel, stressful environment does not play a role.

Potential limitations

Several limitations of this study are noteworthy. First, although we manipulated testosterone exposure in neonatal female mice, it would also be important to know if blocking endogenous testosterone exposure in neonatal males could “feminize” post-UCMS emotionality. This could be accomplished by GDX newborn male mice and measuring emotionality in adulthood. Second, the focus of this study was on the less-investigated developmental origin of sexual dimorphism in emotionality, so the experimental design was not optimized to fully explore potential activational effects of both E₂ and testosterone. Ideally, we would have included Normal Females, Normal Males, and NeoM Females that had been: 1) GDX and implanted with E₂ capsules, 2) GDX and implanted with testosterone capsules, 3) GDX and implanted with blank capsules, 4) sham surgery plus blank capsules, 5) sham surgery plus E₂ capsules, and 6) sham surgery plus testosterone capsules. Due to the experimental constraints on the number of animals that can be used in a UCMS and behavioral study, we had to reasonably select two adult hormone treatment groups to optimize the current study. One of these adult treatment groups needed to be the sham surgery (plus blank capsule) group to allow for comparison to our previous study showing post-UCMS sexual dimorphism of emotionality (Guilloux et al., 2011). It is possible that, by comparing sham groups to matched GDX plus E₂ groups, we missed detecting potential activational effects; i.e. circulating endogenous hormones in the sham groups could have reached a threshold for activational effects similar to the GDX plus E₂ groups, although comparison of sham and GDX plus E₂ groups was sufficient in this study to detect activational effects on locomotion measures. Additionally, comparison to GDX plus testosterone groups would have uncovered potential androgen specific activational effects. This is an important consideration, since normal males have higher adult circulating testosterone levels than females. Future studies focused on the activational effects of hormones should then include comparisons of GDX plus blank mice to GDX plus E₂ or testosterone within each neonatal treatment group to more fully test for the effects of circulating E₂ and testosterone on emotionality.

CONCLUSIONS

Our current results demonstrate that the increased female vulnerability of adult mice to develop high emotionality can be partially reduced (i.e. masculinized) by manipulating the organizational actions of sex hormones early in life. Translating these findings to human counterparts would suggest that the sex difference in MDD prevalence and susceptibility to develop MDD episodes could be partially influenced during development by hormone exposure. Sex differences in developmental testosterone exposure in humans could organize anatomy, networks, and/or gene expression in brain regions involved in mood regulation, resulting in a higher female vulnerability to develop mood disorders. Future studies may benefit from the relative conservation of amygdala transcriptome in mice and humans (Lin et al., 2011; Sibille et al., 2009) and from the use of mice for whom gonadal sex has been dissociated from genetic sex (i.e. the FCG mice) to further assess the relative contribution of organizational and activational effects of hormones, and of sex chromosome complement in establishing the molecular, cellular, and anatomical substrates of sex differences in affect regulation and risk for psychopathology in humans. A better understanding of the mechanisms underlying these sex differences could have profound consequences for sex-specific treatment or even prevention of MDD and other mood disorders.

Highlights

- Neonatal testosterone partially masculinizes adult stress-induced emotionality
- Adult circulating E₂ does not influence stress-induced emotionality
- Locomotion is masculinized by neonatal testosterone and revealed by adult E₂

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

BNSTp	principal nucleus of the bed nucleus of the stria terminalis
E₂	estradiol
EPM	elevated Plus Maze
FCG	four core genotype
FST	forced swim test
GDX	gonadectomy
MDD	major depressive disorder
NSF	novelty suppressed feeding
OF	open Field
PCA	principal component analysis
PMDD	premenstrual dysphoric disorder
P0	postnatal day 0
SEM	standard error of the mean
TP	testosterone propionate

UCMS unpredictable chronic mild stress

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Figure 1.

Study design. (A) On postnatal day zero (P0), females were injected with either TP or vehicle (peanut oil) and males were injected with vehicle. At 5 months of age, mice were GDX and implanted with E₂ capsules or given sham surgery and blank implants. At 6 months of age, baseline emotion and locomotion of mice were assessed using the EPM, OF, and NSF tests. Mice were then exposed to 8 weeks of UCMS. UCMS-induced emotionality and locomotion were assessed during the last two weeks of the UCMS protocol using the EPM, OF, NSF, and FST tests. (B) Example graph illustrating group comparisons (See methods).

Figure 2. Effect of (A) neonatal TP on BNSTp volume and (B) adult hormone manipulation on E₂ serum levels. **, p < 0.01; ***, p < 0.001; #, p < 0.1.

Figure 3. Effects of neonatal TP on baseline emotionality behavior. *, p < 0.05; **, p < 0.01. “Adult hormones” refer to a main activational effect.

Figure 4.

Effects of neonatal TP on baseline locomotor behavior. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Black asterisks indicate comparisons between neonatal treatment groups and grey asterisks indicate within neonatal treatment group comparisons of Shams and E-Clamps.

Figure 5.

Effects of neonatal TP on post-UCMS emotionality behavior. *, $p < 0.05$; **, $p < 0.01$; #, $p < 0.1$. “Adult hormones” refer to a main activational effect.

Figure 6.
 Effects of neonatal TP on post-UCMS Locomotion.

Table 1

Treatment and size of experimental groups

Neonatal Treatment	Adult Surgery	Group Name	Baseline N	Post-UCMS N
Female				
Vehicle	Sham + Blank	Normal Female/Sham	10	10
	GDX + E2	Normal Female/E-Clamp	13	13
TP	Sham + Blank	NeoM Female/Sham	15	14
	GDX + E2	NeoM Female/E-Clamp	9	7
Male				
Vehicle	Sham + Blank	Normal Male/Sham	18	17
	GDX + E2	Normal Male/E-Clamp	9	6

Table 2

Statistical values associated with baseline emotionality (top) and locomotion (bottom) measures.

Dependent measure	Main organizational effect	Main activation effect	Neonatal-by-adult hormone interaction	Organizational /Sex posthoc	Organizational × Activation interaction posthoc
Time in open arms (EPM)	F = 0.19; df = 2 p > 0.8	F = 1.13; df = 1 p > 0.25	F = 1.50; df = 2 p > 0.2	N/A	N/A
% Crosses into open arms (EPM)	F = 0.62; df = 2 p > 0.5	F = 3.03; df = 1 p = 0.09	F = 2.04; df = 2 p > 0.1	N/A	N/A
Time in center (OF)	F = 1.67; df = 2 p = 0.2	F = 3.64; df = 1 p = 0.06	F = 1.29; df = 2 p > 0.25	N/A	N/A
% Distance in open (OF)	F = 5.07; df = 2 p < 0.01	F = 0.46; df = 1 p = 0.50	F = 0.67; df = 2 p > 0.5	Normal F < Normal M (p < 0.03) Normal F = NeoM F (p > 0.15) NeoM F < Normal M (p < 0.01)	N/A
Latency to eat (NSF)	F = 0.59; df = 2 p > 0.5	F = 7.93; df = 1 p < 0.01	F = 1.13; df = 2 p > 0.3	N/A	N/A
Baseline emotionality (Z)	F = 2.10; df = 2 p > 0.1	F = 0.16; df = 1 p > 0.6	F = 0.56; df = 2 p > 0.5	N/A	N/A
Total crosses (EPM)	F = 9.48; df = 2 p < 0.001	F = 2.42; df = 1 p > 0.1	F = 1.00; df = 2 p > 0.35	Normal F > Normal M (p < 0.02) Normal F > NeoM F (p < 0.001) NeoM F = Normal M (p > 0.15)	Normal F Sham = E-Clamp (p > 0.15) NeoM F Sham > E-Clamp (p < 0.06) Normal M Sham = E-Clamp (p > 0.50)
Total distance (OF)	F = 8.55; df = 2 p < 0.001	F = 0.45; df = 1 p > 0.5	F = 4.12; df = 2 p < 0.03	Normal F > Normal M (p < 0.001) Normal F = NeoM F (p > 0.9) NeoM F > Normal M (p < 0.01)	N/A
Baseline locomotion (Z)	F = 10.47; df = 2 p < 0.001	F = 1.31; df = 1 p > 0.25	F = 3.25; df = 2 p < 0.05	Normal F > Normal M (p < 0.001) Normal F = NeoM F (p > 0.15) NeoM F > Normal M (p < 0.01)	Normal F Sham = E-Clamp (p > 0.4) NeoM F Sham > E-Clamp (p < 0.05) Normal M Sham = E-Clamp (p > 0.8)

Table 3

Statistical values associated with post-UCMS emotionality (top) and locomotion (bottom) measures.

Dependent measure	Main organizational effect	Main activation effect	Neonatal-by-adult hormone interaction	Organizational /Sex posthoc	Organizational × Activation interaction posthoc
Time in open arms (EPM)	F = 8.08; df = 2 p < 0.005	F = 0.33; df = 1 p > 0.50	F = 1.27; df = 2 p > 0.25	Normal FeM < Normal M (p < 0.005) Normal FeM < NeoM FeM (p < 0.1) NeoM FeM < Normal M (p < 0.05)	N/A
% Crosses into open arms (EPM)	F = 5.80; df = 2 p < 0.01	F = 0.01; df = 1 p > 0.92	F = 0.30; df = 2 p > 0.7	Normal FeM < Normal M (p < 0.01) Normal FeM = NeoM FeM (p > 0.15) NeoM FeM < Normal M (p < 0.05)	N/A
Time in center (OF)	F = 1.01; df = 2 p > 0.35	F = 6.82; df = 1 p < 0.05	F = 0.45; df = 2 p > 0.60	N/A	N/A
% Distance in open (OF)	F = 1.04; df = 2 p > 0.35	F = 3.18; df = 1 p < 0.1	F = 0.42; df = 2 p > 0.65	N/A	N/A
Latency to eat (NSF)	F = 1.53; df = 2 p > 0.2	F = 3.99; df = 1 p = 0.05	F = 0.51; df = 2 p > 0.6	N/A	N/A
Time swimming (FST)	F = 0.65; df = 2 p > 0.5	F = 6.84; df = 1 p < 0.05	F = 1.39; df = 2 p > 0.25	N/A	N/A
Post-UCMS emotionality (Z)	F = 6.64; df = 2 p < 0.005	F = 0.07; df = 1 p > 0.75	F = 0.44; df = 2 p > 0.6	Normal FeM > Normal M (p < 0.005) Normal FeM > NeoM FeM (p < 0.04) NeoM FeM > Normal M (p = 0.72)	N/A

Dependent measure	Main organizational effect	Main activational effect	Neonatal-by-adult hormone interaction	Organizational /Sex posthoc	Organizational × Activational interaction posthoc
Total crosses (EPM)	F = 0.044; df = 2 p > 0.95	F = 1.74; df = 1 p > 0.15	F = 1.66; df = 2 p > 0.15	N/A	N/A
Total distance (OF)	F = 0.23; df = 2 p > 0.75	F = 3.01; df = 1 p < 0.1	F = 2.31; df = 2 p > 0.1	N/A	N/A
Post-UCMS locomotion (Z)	F = 0.084; df = 2 p > 0.90	F = 1.95; df = 1 p > 0.15	F = 1.79; df = 2 p > 0.15	N/A	N/A