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Sex-specific neuroendocrine and behavioral phenotypes in hypomorphic Type II Neuregulin 1 rats

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1. Introduction

Neuregulin 1 (NRG1) is a growth factor that plays many roles in central nervous system development and function. In both humans and rats, the *Nrg1* gene is highly complex and is alternatively spliced into six types of proteins, all of which contain and signal via an epidermal growth factor (EGF) domain [1-3]. The effects of NRG1 are mediated by the ErbB tyrosine kinase receptor family, particularly ErbB4 [1]. NRG1 and ErbB4 signaling has been implicated in synapse formation, radial neuron migration, synaptic plasticity, neurotransmitter receptor expression and the hormonal control of puberty [1-4]. Furthermore, NRG1 is expressed in many regions of the brain, including the hippocampus (HPC), prefrontal cortex (PFC) and paraventricular nucleus (PVN) of the hypothalamus [5,6]. Additionally, *NRG1* has repeatedly been identified as a susceptibility gene for schizophrenia and is also associated with bipolar disorder [3,7-9]. This strong association with schizophrenia has prompted investigation of the functional role of NRG1 and ErbB4 with mutant animal models.

Recently, we have shown that NRG1 may also be involved in regulating hypothalamic-pituitary-adrenal (HPA) axis activity [6]. The HPA axis facilitates adaptation to both internal and environmental stressors. Adrenal glucocorticoid (GC) hormones (such as corticosterone in the rat) are the principal mediators of this adaptive response. GCs influence the activity of neurons in the brain by binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which regulate neuronal gene transcription and metabolism and can modulate complex behaviors [10-13]. In hypomorphic Type II NRG1 (*Nrg1Th*) male rats, we found disruptions in basal and acute stress recovery corticosterone (CORT) secretions as well as differential changes in expression of GR receptors in the brain [6]. The involvement of NRG1 in the regulation of the HPA axis may be relevant in its role as a schizophrenia susceptibility gene as stressful events, alterations in GC secretion or GR and MR function are associated with multiple psychiatric disorders [10,14-19].

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Several *Nrg1* knock out (KO) mouse lines have been behaviorally characterized and exhibit different phenotypes depending on the region knocked out. In these mice, mutations have typically been targeted to 3' regions of the NRG1 gene, including the transmembrane (TM) domain [7,20,21], the EGF domain [22] and immunoglobulin-like (Ig) domain [23]. However, it is the 5' region of *NRG1* in humans that contains the most risk haplotype associations with schizophrenia and bipolar disorder [3,7,8]. The 5' region codes for two isoforms of NRG1, Type IV and Type II, though Type IV may not be expressed at a detectable level in the adult rat brain [24,25]. In heterozygous *Nrg1* TM domain KO mice, hyperactivity, failure to habituate and impaired prepulse inhibition (PPI) are reported [7,21,26,27]. While heterozygous *Nrg1* EGF domain KO mice also demonstrate hyperactivity, they show a facilitated ability to habituate to a novel environment [28], whereas heterozygous *Nrg1* Ig domain KO mice do not exhibit a hyperactive phenotype [23]. Mice over-expressing Type I NRG1 or with decreased expression of Type III NRG1 also demonstrate PPI deficits [29,30]. We have shown that male rats homozygous for the *Nrg1*^{Tn} mutation are not hyperactive and fail to habituate to an open field [6].

As is typical of animal research, relatively few studies of *Nrg1* mutant mice have been conducted with females. However, those that did include females found several sex-specific differences in exploration of environment, spatial cognition and anxiety behavior caused by disruption of NRG1 [21,26,29]. Thus it is feasible that NRG1 may play a role in regulating certain behaviors in a sex-specific manner. Additionally, NRG1-ErbB4 signaling in hypothalamic astrocytes is required for normal female sexual development [4]. Mice expressing dominant-negative ErbB4 receptors displayed delayed sexual maturation and diminished reproductive capacity in adulthood as a result of the inability of hypothalamic astrocytes to respond to NRG1 [4]. It is currently unknown whether disruptions to NRG1 itself result in altered female sexual development or specific measures of adult reproductive capability. However, evidence from several heterozygous *Nrg1* mutant mouse lines supports that *Nrg1* mutant mice are viable and fertile [22,7,26].

The primary goal of these studies was to test the hypothesis that NRG1 plays a role in regulating sexually dimorphic neuroendocrine and behavioral responses to the environment. To accomplish this goal, female *Nrg1*^{Tn} rats were used to determine the effects of disrupted Type II NRG1 on pubertal onset, adult reproductive capability, basal and stress induced CORT secretion and GR and MR expression. In the present study, open field locomotor activity and habituation were also examined in females, while acoustic startle and PPI were examined in both males and females. In addition, potential differences in these phenotypes between male and female *Nrg1*^{Tn} rats were explored.

2. Methods and Materials

2.1. Animals

Random cycling female and male (acoustic startle and PPI studies only) Fischer 344 wild type (WT) and homozygous *Nrg1* mutant (*Nrg1*^{Tn}) rats were used in the present studies. This model was developed and obtained from the PhysGen Program in Genomic Applications (<http://pga.mcw.edu/>) at the Medical College of Wisconsin. In these animals, the mRNA and protein corresponding to Type II NRG1 in the brain are disrupted by the random insertion of the Sleeping Beauty transposon into the first intron of the *Nrg1* gene [6]. All animals were housed at the Maryland Psychiatric Research Center in a temperature- and light-controlled (lights on 06.00 h to 20.00 h) facility. Water and chow (Harlan Teklad, Frederick, MD) were available *ad libitum*. All procedures conformed to the guidelines for animal research established by the National Institutes of Health, and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Separate groups of animals were used for each set of reproductive measurements, acute stress and western blotting and each behavioral experiment.

2.2. Female pubertal onset and adult reproductive function

2.2.1. Pubertal onset—Starting on postnatal day 29, female WT and *Nrg1^{Tn}* rats (n=8-9/genotype) were inspected daily for inperforation of the vaginal membrane (i.e. vaginal opening) as a marker for the onset of puberty. In female rats, ovulation and the hormonal changes that accompany the onset of puberty are usually associated with vaginal opening [31].

2.2.2. Adult reproductive function—Adult (~ 65 days of age) female WT and *Nrg1^{Tn}* rats (n=10-13/genotype) were used for determination of estrous cyclicity. Using the lavage method [32,33], vaginal samples were taken once a day for 15 days between 11.00 h and 14.00 h. Samples were observed under a light microscope at 100 x magnification. The proportion of cell types was used to determine estrous cycle phases as follows: proestrus consisted of primarily round nucleated epithelial cells, estrus was characterized by predominantly cornified epithelial cells, and diestrus I and II were characterized by a predominance of leukocytes [32,33].

To determine adult reproductive capacity, a group of nulliparous female WT and *Nrg1^{Tn}* rats (n=10-12/genotype) the interval between initial exposure to a male breeder and the birth of the first litter was recorded. In addition, litter size, percent survival from birth to weaning and percent male pups for these litters were recorded and compared between genotypes.

2.3. Neuroendocrine Measurements

2.3.1. Acute restraint stress—Adolescent (43-45 days of age) and adult (76-79 days of age) female WT and *Nrg1^{Tn}* (n=43-46/genotype) rats were moved from the vivarium to a temperature- and light-controlled behavioral testing room for acclimation. Approximately 65 % of these females (and subsequent plasma samples) were run simultaneously with the male rats described in our previous publication [6] and additional animals were added at a later time. Animals were handled and exposed to acute restraint stress as previously described [6]. Two weeks after being moved to the behavioral testing room, animals were randomly divided into three groups and each group was euthanized by decapitation at a different time point: before (basal time point, n=16-19/genotype), at the end of (peak time point, n=13/genotype) or 120 minutes after (recovery time point, n=14/genotype) exposure to an acute restraint stress session. Acute restraint stress consisted of placing the animals in cylindrical plastic restraint tubes for 30 minutes according to our previously published protocol [34]. Trunk blood was collected into tubes containing 10% EDTA and plasma was isolated by centrifugation. Brains and pituitary glands were rapidly removed from the skull and frozen on powdered dry ice. Both brains and plasma were stored at -80°C until use. Plasma levels of CORT were determined by radioimmunoassay according to protocols provided by the manufacturer (MP Biomedicals, Orangeburg, NY).

2.3.2. Western blotting—Western blots were performed as previously described [6] with the following modifications. Whole brains and pituitary glands (n=11/genotype) were extracted from the skull of adult female WT and *Nrg1^{Tn}* rats at the basal time point of the acute stress experiment (section 2.3.1) to examine GR and MR expression in the dorsal HPC (n=8/genotype), PVN (n=8/genotype) and amygdala (AMG) (n=8/genotype). Brain regions were identified using the Paxinos and Watson brain atlas [35]. Brain tissue samples from the HPC, PVN and AMG were isolated from 1mm frozen coronal sections using a 1mm rat brain punch (Stoelting, Wood Dale, IL). Bilateral punches were used for each area with a

total of 6 punches for dorsal HPC, 2 punches for the PVN and 4 punches for the AMG. Some tissue adjacent to the PVN was likely included in these punches.

Tissue samples were homogenized, protein concentrations were determined, diluted, resolved by electrophoresis, electrotransferred and blocked as previously described [6]. After blocking, membranes were incubated with the appropriate primary antibody for 24 or 48 h at 4°C: GR (BuGR2, 1:1000; ab2768, Abcam, Cambridge, MA) and MR (1:1000; sc-11412, Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated with the appropriate horseradish peroxidase-labeled secondary antibody for 1 h at room temperature: goat anti-rabbit (1:2500; AP307P, Millipore, Temecula, CA) and goat anti-mouse (1:5000; AP124P, Millipore, Temecula, CA). Membranes were then washed and the protein detected using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA). Blots were developed by exposure to chemiluminescent film (Amersham, GE Healthcare, Buckinghamshire, UK). Densitometric measurements of the protein bands were made using AIS 6.0 software (Imaging Research Inc., St. Catharines, ON, Canada). Following image collection, membranes were stripped using Restore plus Western blot stripping buffer (Thermo Fisher Scientific, Waltham, MA) and reprobed for MR (HPC and AMG only) and actin (1:10,000; MAB1501, Millipore, Temecula, CA). Protein expression levels were normalized to actin expression. Bands were chosen for densitometric analysis based on molecular weight per manufacturer specifications as follows, GR: 97 kDa, MR: 102 kDa. Sex differences were not examined in MR and GR expression due to the fact that blots for males and females were run separately and differences in blot exposures could confound the interpretation of any significant findings. Two WT pituitary samples, one *Nrg1^{Tn}* PVN sample and one WT AMG sample were identified as statistical outliers using the Grubbs test; therefore these animals were dropped from the western blot analyses.

2.4. Behavioral Measurements

2.4.1. Open field testing and habituation—Adult female WT and *Nrg1^{Tn}* rats (n=8-12 per group) were moved from the vivarium to the behavioral testing room and allowed to acclimate for 30 minutes each day before testing. Locomotor activity of the rats was evaluated in an open field arena for 10 minutes on two consecutive days. The arena was a 62 × 62 × 46 cm box with black Plexiglas walls. The floor of the arena was covered with unscented cat litter to allow for easy removal of urine and feces between each animal. Locomotor activity was recorded and scored using an ANY-maze video tracking system (version 4.6, Stoelting, Wood Dale, IL). Time mobile, line crossing and distance traveled were the indices chosen for analysis. In addition, to determine within-session habituation, the differences in locomotor activity between the first and second half of the first exposure to the open field were calculated as a percent decrease in activity. To examine sex differences in locomotor activity an overall locomotor activity index was generated by converting each subject's raw score on each index to a T-score (10*(Z-score) +50) and then averaging the three T scores. One *Nrg1^{Tn}* rat was identified as a statistical outlier for locomotor activity using the Grubbs test and was dropped from the open field study.

2.4.2. Acoustic startle response and prepulse inhibition—An automated startle reflex system (SR Lab, San Diego Instruments, San Diego, CA) was used to measure the acoustic startle response (ASR) and PPI in adult female (n=7 per genotype) and male (n=8 per genotype) animals as previously described [34]. Individual startle trials (120 dB for 40 ms) were used to measure baseline startle response. The trials in which a weak prepulse stimulus (1, 3, 6, 12 or 15 dB above background for 20 ms) were followed by a 120 dB startle stimulus assessed sensorimotor gating. The ability of the preceding stimulus to attenuate the startle response to the 120 dB stimulus is termed PPI. Background noise (65 dB) was present during the entire 22-minute trial. A total of 23 individual startle trials, 8 of

each 1 and 15 dB, and 9 of each of the 3, 6 and 12 dB PPI trials were presented in a pseudo-randomized order. The inter-trial interval was 15 ms. Holding cylinders within the isolation chambers were cleaned with 75 % ethanol between each session. Groups were counterbalanced to ensure that each experimental group had equal exposure to each chamber. Baseline acoustic startle is expressed as the average of all acoustic startle trials (120 db) except for the first block of trials. Habituation to acoustic startle was determined by expressing the difference in response between the first and last block of startle trials as a percent decrease in response. PPI is presented as the percent decrease in startle amplitude as a function of the magnitude of the prepulse stimulus using the following formula: percent decrease = $100 - ([\text{prepulse startle}] / [\text{average of stimulus alone}] \times 100)$.

2.5. Statistical methods

Statistical analyses were conducted using SPSS statistical software (version 12.0). Unpaired t-tests were used to compare mean group differences in pubertal onset and adult reproductive function studies. To analyze CORT data obtained by RIA, analysis of variance (ANOVA) was used. CORT levels did not differ at the time points measured between adolescent and adult WT or *Nrg1^{Tn}* rats, nor was there an age by genotype interaction (data not shown). Thus, CORT data for adolescent and adult rats were combined for subsequent analysis. Unpaired t-tests were used to compare mean protein expression in females. For the open field test, repeated measures ANOVAs were conducted, with trial included as a repeated measures factor. To compare habituation on the first day of open field exposure, unpaired t-tests were used. ANOVAs were used to compare baseline ASR and ASR habituation. PPI was analyzed with repeated measures ANOVA. To examine sex differences in acute restraint stress and open field behavior, data from our previous study in male WT and *Nrg1^{Tn}* rats [6] were combined with the current study in females for analysis. Examination of sex differences in ASR and PPI was built into the experimental design. Post hoc tests, conducted using I-matrix contrast statements in SPSS, were used to follow up any significant interactions or planned comparisons.

3. Results

3.1. Female pubertal onset and adult reproductive function

Disruption of Type II NRG1 in female rats did not affect the timing of puberty, adult estrous cyclicity or reproductive capacity (summarized in Table 1). There was no difference in the age of vaginal opening between WT and *Nrg1^{Tn}* rats. Additionally, mean estrous cycle length was not altered by disruption of Type II NRG1 in Fischer rats. To measure reproductive capacity, the number of days to first litter, the number of pups born, percent survival and percent male at weaning were recorded. There were no differences between WT and *Nrg1^{Tn}* rats in any of these measures.

3.2. Neuroendocrine Measurements

3.2.1. Acute restraint stress—A 2×3 ANOVA (Genotype (WT vs. *Nrg1^{Tn}*) × Time (basal vs. peak vs. recovery)) revealed a significant effect of time ($F_{2,83} = 231.298$, $p < 0.001$; Fig. 1). Regardless of genotype, all animals mounted a stress response after an acute restraint stress, with peak CORT levels being significantly greater than basal ($p < 0.001$; Fig. 1) CORT levels. Moreover, 120 minutes following the termination of the stressor, CORT levels were significantly reduced relative to peak CORT levels ($p < 0.001$; Fig. 1) in all groups. Planned comparisons were used to determine genotype differences at each time point. These comparisons revealed that *Nrg1^{Tn}* females exhibited enhanced suppression of CORT at recovery compared to both WT females ($p < 0.02$; Fig. 1) and compared to their own basal CORT levels ($p < 0.02$; Fig. 1). No differences between genotypes were detected at

the basal or peak time point, and CORT levels for WT females were equivalent between the basal and recovery time points.

Data from males and females were combined in a $2 \times 2 \times 3$ ANOVA (Sex (male vs. female) $\times$ Genotype (WT vs. *Nrg1^{Tn}*) $\times$ Time (basal vs. peak vs. recovery)). This analysis confirmed the aforementioned main effect of time ($F_{2,168} = 571.08$, $p < 0.001$) and the interaction between time and genotype ($F_{2,168} = 3.62$, $p < 0.03$). An effect of sex was also revealed ($F_{1,168} = 49.89$, $p < 0.001$) such that females had higher CORT levels than males. Finally, an interaction between time point and sex was detected ($F_{2,168} = 13.303$, $p < 0.001$). Planned comparisons were used to compare within-genotype sex differences at each time point. At the basal time point, WT female CORT levels were significantly higher than WT male levels ($p < 0.01$; Fig. 2A), while there was no significant difference between *Nrg1^{Tn}* males and females. An effect of genotype was also observed at this time point with *Nrg1^{Tn}* rats, regardless of sex, showing higher CORT levels than WT rats ($p < 0.03$; Fig. 2A). At the peak time point, a sex difference was found between both WT and *Nrg1^{Tn}* males and females ($p < 0.01$, $p < 0.001$ respectively; Fig. 2B), with females of both genotypes having higher CORT levels than males. Finally, at the recovery time point, 120 min after removal from the restraint tubes, WT females had significantly higher CORT than WT males ($p < 0.02$; Fig. 2C) whereas *Nrg1^{Tn}* males and females were indistinguishable. At this time point, an effect of genotype was also observed, with *Nrg1^{Tn}* rats, regardless of sex, showing lower CORT levels than WT rats ($p < 0.04$; Fig. 2C).

3.2.2. Glucocorticoid and mineralocorticoid receptor—Results for GR and MR protein expression in female *Nrg1^{Tn}* rats are summarized in Table 2. Adult female *Nrg1^{Tn}* rats showed reduced expression of GR in the pituitary ($p < 0.01$; Table 2) compared to WT rats. No difference in GR expression between WT and *Nrg1^{Tn}* females was found in the PVN. In the HPC and AMG, samples were probed for both GR and MR. No differences in GR or MR expression were found between WT and *Nrg1^{Tn}* females in the HPC or AMG. However, in the AMG, a decreased MR/GR ratio was found in female *Nrg1^{Tn}* rats compared to WT rats, though the significance of this decrease became a trend after Welch's correction was used to account for the different variances between groups ($p \leq 0.07$; Table 2).

3.4. Behavioral Measurements

3.4.1. Open field testing and habituation—Time mobile, number of line crossings and distance traveled were utilized as indices of locomotor activity during the open field testing in female rats. 2×2 repeated measures ANOVAs (Genotype (WT vs. *Nrg1^{Tn}*) $\times$ Trial (exposure 1 vs. exposure 2)) were conducted on these indices in females, with trial included as the repeated measure. These analyses revealed that time spent mobile ($F_{1,17} = 16.29$, $p < 0.01$; Fig. 3A), number of line crossings ($F_{1,17} = 7.85$, $p < 0.02$; Fig. 3B) and distance traveled ($F_{1,17} = 11.27$, $p < 0.01$; Fig. 3C) decreased with repeated exposure to the open field arena in both groups. Thus, both WT and *Nrg1^{Tn}* females habituated to the open field. However, *Nrg1^{Tn}* females spent less time mobile ($F_{1,17} = 6.73$, $p < 0.02$; Fig. 3A), had fewer line crossings ($F_{1,17} = 5.99$, $p < 0.03$; Fig. 3B) and traveled less distance ($F_{1,17} = 4.85$, $p < 0.05$; Fig. 3C) compared to WT females across exposures to the open field. To determine if decreased locomotor activity was indicative of anxiety, within-session habituation was determined for the first exposure to the open field. Upon first exposure to the open field, *Nrg1^{Tn}* females demonstrated more pronounced within-session habituation in locomotor activity compared to WT females on all indices (time mobile: $p < 0.01$; line crossing: $p < 0.001$; distance traveled: $p < 0.001$; Fig. 3D-F respectively).

To examine sex differences, the locomotor index (average T-score of all three indices) was analyzed with a $2 \times 2 \times 2$ repeated measures ANOVA (Sex (male vs. female) $\times$ Genotype (WT

vs. *Nrg1^{Tn}* × Trial (exposure 1 vs. exposure 2)) with trial included as the repeated measure. This analysis revealed a significant effect of sex ($F_{1,41} = 17.02$, $p < 0.001$; Fig. 4) and a significant interaction between sex and genotype ($F_{1,41} = 8.36$, $p < 0.01$; Fig. 4). No effect of exposure was found, therefore the two days were combined for presentation in Figure 4. Post hoc analysis revealed a sex difference in *Nrg1^{Tn}* rats ($p < 0.001$; Fig. 4) on overall locomotor activity, whereas there was no sex difference in WT rats. Unexpectedly, the sex difference in *Nrg1^{Tn}* rats on locomotor activity was consistent with females being less active than males.

3.4.2. Acoustic startle response and prepulse inhibition—A 2×2 ANOVA (Genotype (WT vs. *Nrg1^{Tn}*) × Sex (Male vs. Female)) revealed a significant effect of sex ($F_{1,26} = 6.88$, $p < 0.02$; Fig. 5A) on baseline ASR, with males exhibiting greater startle amplitude than females. To examine habituation, a 2×2 ANOVA (Genotype (WT vs. *Nrg1^{Tn}*) × Sex (Male vs. Female)) was utilized on the percent decrease in ASR. This analysis revealed a significant effect of genotype ($F_{1,26} = 7.6$, $p < 0.02$; Fig. 5B), with *Nrg1^{Tn}* rats displaying enhanced habituation compared to WT rats.

To analyze PPI, a 2×2×4 repeated measures ANOVA (Genotype (WT vs. *Nrg1^{Tn}*) × Sex (male vs. female) × Prepulse intensity (3dB vs. 6dB vs. 12dB vs. 15 dB)) was used. The 1dB prepulse was excluded from analysis as it resulted in prepulse facilitation in all groups. This analysis revealed a main within-subjects effect of prepulse intensity ($F_{3,78} = 119.3$, $p < 0.001$; Fig. 5C) in all groups, such that higher dB prepulses resulted in increased PPI. Between-subject effects included a main effect of genotype ($F_{1,26} = 6.78$, $p < 0.02$; Fig. 5C) and an interaction between genotype and sex ($F_{1,26} = 4.38$, $p < 0.05$; Fig. 5C). Post hoc analyses revealed that the female groups drove the main effect of genotype, whereby WT females demonstrated more robust PPI than *Nrg1^{Tn}* females ($p < 0.03$; Fig. 5C) while WT and *Nrg1^{Tn}* males were indistinguishable ($p = 0.63$; Fig. 5C). A sex difference in PPI was not detected between either WT males and females or *Nrg1^{Tn}* males and females (Fig. 5C).

4. Discussion

4.1. Summary of Results

Disruption of Type II NRG1 in female rats led to alterations in HPA axis function, locomotor activity and sensorimotor gating, without affecting measures of pubertal onset and adult reproductive capability. No differences between WT and *Nrg1^{Tn}* rats were observed for age of vaginal opening or estrous cycle length, or in any measure of reproductive capability or litter characteristics. However, *Nrg1^{Tn}* female rats demonstrated enhanced suppression of CORT secretion after recovery from a 30 minute stress, compared to WT females. In addition, *Nrg1^{Tn}* female rats displayed reduced GR expression in the pituitary and a slight reduction in the MR/GR ratio in the AMG. In the open field arena, *Nrg1^{Tn}* female rats were less active than WT females, but exhibited enhanced habituation during the first exposure. Both male and female *Nrg1^{Tn}* rats exhibited enhanced habituation to acoustic startle, while only female *Nrg1^{Tn}* rats displayed a reduction in PPI, a measure of sensorimotor gating. Furthermore, disruption of Type II NRG1 altered normal sex differences in several assays.

4.2. Female pubertal onset and adult reproductive function

Normal female sexual development has been shown to depend on proper NRG1-ErbB4 signaling [4]. However, the disruption of Type II NRG1 in the rat brain did not result in delayed sexual maturation and diminished reproductive capacity in adulthood as was found in mice with disrupted ErbB4 [4]. We found that the time of vaginal opening, an external sign of puberty, was the same between WT and *Nrg1^{Tn}* female rats and was also consistent

with other reports in Fischer 344 rats [36]. Additionally, both genotypes had the same average estrous cycle length. It is also known that adult reproductive capability can be altered by compromised ErbB4 function, therefore we examined several relevant indices [4]. Most notably, there was no difference between WT and *Nrg1^{Tn}* female rats in the time it took from the first exposure to a male until the birth of their first litter. This measure accounts for both ability to conceive and gestation length. In addition, no differences between genotypes were found in the number of pups born, the percent of those pups surviving until weaning and the sex ratio of the litters. These findings indicate that a subtle disruption to Type II NRG1 does not affect female reproductive function. This is important, as our other findings in the *Nrg1^{Tn}* females can not be attributed to altered sexual development or function, but rather to other aspects in the regulation of sex-specific behaviors. Furthermore, as all *Nrg1^{Tn}* rats are born to *Nrg1^{Tn}* dams, the lack of gross disruptions in litter size, sex ratio, dam reproductive function and maternal behavior [6] represent important findings relevant to the interpretation of the phenotypes exhibited by both male and female offspring.

4.3. Neuroendocrine alterations

Nrg1 mRNA and Type II NRG1 protein are expressed in the HPC, PFC and PVN [6,37]. This localization implicates Type II NRG1 in the regulation of neuroendocrine and behavioral responses to stress. However, the effect of reducing Type II NRG1 expression appears to be of little consequence on basal and restraint stress-induced activation of CORT secretion in female rats since there is no difference between CORT responses in WT and the *Nrg1^{Tn}* rats under these conditions. On the other hand, our studies did reveal an overshoot in the deactivation of CORT secretion after restraint stress resulting in significantly reduced post-stress recovery CORT levels in female *Nrg1^{Tn}* rats. This finding is consistent with observations in male rats with the same genetic alteration, though, unlike females, male *Nrg1^{Tn}* rats display significantly increased basal CORT secretion [6].

Enhanced suppression of CORT secretion after recovery from an acute stress is a rare occurrence in HPA axis physiology but does occur in pathological situations, such as post-traumatic stress disorder [38,39]. The enhanced suppression of CORT following stress would commonly be coupled with an up-regulation of GR levels in the pituitary, PVN or HPC. But contrary to this expectation, GR expression is relatively unaffected in the brains of female *Nrg1^{Tn}* rats and is even reduced in the pituitary, which is, indeed, paradoxical. Although, it should be noted that GR expression in the AMG is slightly increased which led to a reduced MR/GR ratio. While the AMG is certainly involved in modulating HPA axis function [40] it is not clear how this subtle change could cause a significant enhancement of GC negative feedback in this instance. However, dissociations between neuroendocrine stress responses and GR expression have been reported [41-44] and indicate that other mechanisms may be involved in this response.

One potential mechanism underlying enhanced suppression of post-stress CORT release could be a compromised ability to reactivate normal stimulatory neuropeptide drive for HPA axis secretions after stress. This deficit could be due to altered regulation of corticotropin-releasing hormone or arginine vasopressin expression, secretion or signaling [45-47]. Alternatively, an imbalance in the excitatory or inhibitory drive to the neurons of the PVN could also account for the enhanced suppression of post-stress CORT secretion [46]. A more likely explanation for this finding may lie in the known biology of NRG1. Studies have shown that NRG1 is necessary for the establishment of excitatory synapses in GABAergic interneurons and for the development of balanced excitatory/inhibitory tone in the brain via induction of activity-dependent GABA release [48,49]. It is clear from several studies that both glutamatergic and GABAergic mechanisms play a role in controlling HPA axis function [50,51] and disruption of metabotropic glutamate receptor expression produces

enhanced post-stress suppression of CORT [52]. While further studies will be needed to confirm the presence of an altered balance between glutamatergic and GABAergic tone in the *Nrg1^{Tn}* rats, the behavioral findings presented in this report would also tend to support this proposition, as disruptions in glutamate and GABA function lead to PPI deficits [48,53].

4.4. Behavioral Reactivity

4.4.1. Open field testing and habituation—Female *Nrg1^{Tn}* rats demonstrated decreased locomotor activity on all indices measured in the open field test. Findings in females of *Nrg1* mutant mouse models vary with the nature of the disruption to NRG1. Female mice over-expressing Type I NRG1 were less active in the open field [29], while female mutant TM domain NRG1 mice exhibited increased locomotor activity during initial exploration [26]. The present findings in female *Nrg1^{Tn}* rats are contrary to those found in male *Nrg1^{Tn}* rats, which show normal locomotor activity [6]. While low levels of locomotor activity are sometimes used to infer increased anxiety [54,55], an alternative interpretation may be that decreased locomotor activity reflects decreased agitation in the open field [56]. Interestingly, no difference was found between female WT and *Nrg1^{Tn}* rats in between-session habituation to the open field arena, with both genotypes exhibiting decreased activity on the second exposure compared to the first. In further support of this alternative interpretation, female *Nrg1^{Tn}* rats demonstrate enhanced within-session habituation on the first day. Thus in female rats, disruption of NRG1 may lead to decreased sensitivity to novelty and ultimately, enhanced habituation.

In contrast, male *Nrg1^{Tn}* rats fail to habituate between the first and second exposure to the open field [6]. Mutant TM domain NRG1 mice also display sex-specific differences in habituation of exploratory behavior [26]. Interestingly, the interpretation of decreased locomotor activity as a reflection of decreased agitation would be consistent with the observed enduring locomotor activity in male *Nrg1^{Tn}* rats as an indication of increased agitation. These sex-specific changes in locomotor activity and environmental habituation may be predictive of anxiety-like behavior in a more definitive assay, such as the elevated plus maze.

4.4.2. Acoustic startle response and prepulse inhibition—Baseline ASR can be used as an index of aversive emotional states in animals [57]. Regardless of genotype, male rats exhibited greater ASR than female rats. This is consistent with other reports that male rodents demonstrate increased ASR compared to female rodents [58-61]. What is more interesting is that both male and female *Nrg1^{Tn}* rats demonstrated enhanced habituation to the startle stimulus across the entire session. This is consistent with enhanced within-trial, and normal between-trial habituation found in female *Nrg1^{Tn}* rats in the open field but contrary to the failure to habituate to the open field found in male *Nrg1^{Tn}* rats. Therefore disruption of NRG1 in the rat brain has task- and sex-specific effects on habituation to environmental stimuli.

On the PPI trials, male WT and *Nrg1^{Tn}* rats displayed equivalent PPI, while *Nrg1^{Tn}* females showed reduced PPI compared to WT females. Thus disruption of Type II NRG1 results in a sex-specific effect on PPI as well. Additionally, the PPI deficit in *Nrg1^{Tn}* females is not likely due to altered auditory function, as startle amplitude to individual prepulse tones (1, 3, 6, 12 and 15 dB above 65dB background) was assessed and found to be equivalent between genotypes (data not shown) [62]. Interestingly, both male and female mice over-expressing Type I NRG1 demonstrated a PPI deficit with increased baseline acoustic startle [29], and other studies in TM domain and Type III NRG1 mutant mice, which only included males, found deficits in PPI [7,30]. In addition, a missense mutation in the 5' region of the *NRG1*

gene has been shown to have a possible functional effect on PPI in both control and schizophrenia populations [63].

A reduction in PPI is generally thought to reflect a disruption in sensory gating, such that the brain is inundated with information from environmental stimuli leading to impaired cognitive abilities [64]. However, in light of the aforementioned enhanced habituation, the decrease in PPI in *Nrg1^{Tn}* female rats may also reflect a general decrease in attention to environmental stimuli. One interpretation of this finding is that the female *Nrg1^{Tn}* rats do not perceive the testing environment to be threatening. Several studies have demonstrated that PPI is increased when the environment is perceived as more threatening, such as when shocks are also administered [65,66]. Consistent with our findings in habituation to the open field, female *Nrg1^{Tn}* rats may perceive novel environments as less threatening than WT females, while male *Nrg1^{Tn}* rats may be prone to interpret novel environments as more threatening than WT rats.

4.5. Summary of sex-specific effects

We report here that disruption of Type II NRG1 results in sex-specific phenotypes in basal and recovery CORT secretion, locomotor activity, habituation to an open field, and PPI. It was recently shown that NRG1 increases activity-dependent GABA release [48]. Disruption of GABAergic tone and thus the balance of excitatory and inhibitory neurotransmission throughout development could play a role in mediating some of these sex-specific effects. For example, during early development, GABA acts as an excitatory neurotransmitter and may be important for the masculinization of the brain [67]. Furthermore, males tend to be more sensitive to modulators of the GABA_A receptors [68]. Thus, the normal sexual dimorphism in GABAergic neurotransmission may be altered in different directions by the disruption of Type II NRG1. In addition, ovarian hormones have been shown to effect CORT responses to stress, open field activity and PPI, though the direction of these effects remains complicated and controversial [60,61,69-71]. Estrogen has also been shown to modulate both excitatory and inhibitory states in neurons [72-75]. It may be that circulating gonadal hormones modulate NRG1-induced changes in GABAergic neurotransmission during development and adulthood to produce some of these sex-specific findings. Importantly, we found no alterations between WT and *Nrg1^{Tn}* females in pubertal onset or measures of adult reproductive function, indicating no overt perturbations in ovarian hormone function.

4.6. Disruption of normal sex differences

Several sex-specific findings have already been discussed, but a brief summary of sex differences in WT and *Nrg1^{Tn}* males and females is also warranted (Table 3). Consistent with established findings, WT males have lower CORT concentrations at all time points compared to WT females [76]. However, at the basal and recovery time point, this sex difference is eliminated in the *Nrg1^{Tn}* rats. In overall locomotor activity, no sex difference was found between WT males and females. While this pattern is generally atypical of rats, other studies have also reported a lack of sex difference in the locomotor activity levels of WT Fischer 344 rats [77]. However, an unexpected sex difference emerged in the *Nrg1^{Tn}* rats during open field testing, with male *Nrg1^{Tn}* rats exhibiting more activity than female *Nrg1^{Tn}* rats. On baseline ASR, we found that the sex difference in WT rats was preserved in *Nrg1^{Tn}* rats, with males showing increased startle amplitude compared to females, consistent with reports by others [58-61]. On a final measure, no sex difference between males and females of either genotype was detected on PPI. This lack of sex differences is similar to other reports with males and random cycling females [59,61,78].

As noted in section 2.5, sex differences were examined by comparing of the present data in females with that of our previous study in males [6]. It should be noted that a majority of these females underwent acute stress testing simultaneously with the aforementioned males. In addition, all males and females were run together for the acoustic startle and PPI experiments described here. In the case of open field locomotor activity and habituation, males and females were run on separate occasions. Therefore, some sex differences in CORT levels and open field behaviors could be attributed to minor variances in experimental conditions. However, care was taken to ensure that the experimental procedures were the same for each study, including experimenter, testing time of day and habituation to the testing room. Thus, the likelihood of a systematic experimental error is small.

4.7. Conclusions

The present experiments provide additional evidence for the involvement of Type II NRG1 in the control of HPA axis function, as well as behavioral responses to the environment. In females, disruption of Type II NRG1 resulted in enhanced suppression of CORT following an acute restraint stress. In addition, GC receptor expression was altered in both the pituitary and the AMG. Behaviorally, *Nrg1^{Tn}* females demonstrate reduced locomotor activity and enhanced habituation to both the open field and acoustic startle, as well as reduced PPI. Furthermore, disruption of Type II NRG1 affects males and females differently. We report here sex-specific phenotypic effects in basal and recovery CORT secretion, locomotor activity, habituation to an open field and PPI. These sex-specific phenotypes can not be attributed to female pubertal onset or reproductive capability, indicating that the effects of disrupting NRG1 in females are not directly related to gross changes in the hypothalamic-pituitary-gonadal axis. Moreover, the lack of hypothalamic-pituitary-gonadal axis disruptions in females lends credence to findings in male offspring of *Nrg1^{Tn}* dams. Our results provide further support for the involvement of NRG1 in the control of the neuroendocrine response to stress, the behavioral response to environmental stimuli and the sex-specific nature of this relationship.

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Research Highlights

- Female *Nrg1^{Tn}* rats exhibit normal pubertal onset and reproductive capability
- Female *Nrg1^{Tn}* rats exhibit enhanced neuroendocrine recovery after acute stress
- Unlike male *Nrg1^{Tn}* rats, females show enhanced habituation to novel environments
- Female *Nrg1^{Tn}* rats display reduced locomotion and PPI, whereas males do not
- Disruption of Type II NRG1 results in sex-specific phenotypes

Figure 1.

Female acute restraint stress. Basal, peak and recovery corticosterone (CORT) levels in adult female WT and *Nrg1^{Tn}* rats following a 30 minute restraint stress. Both genotypes mounted a CORT response to the stress with peak CORT levels being significantly greater than basal ($p < 0.001$) and recovery ($p < 0.001$) CORT levels. *Nrg1^{Tn}* females also exhibited enhanced suppression of CORT at the recovery time point, both compared to their own basal levels ($*p < 0.02$) and also compared to WT animals at the recovery time point ($*p < 0.02$). Recovery CORT levels in WT females were equivalent to their own basal CORT levels. Bars represent mean $\pm$ SEM.

Figure 2.

Sex differences in CORT secretion. At the basal time point (A), WT female CORT levels were significantly greater than WT male CORT (** $p < 0.01$) while no sex difference was detected in *Nrg1^{Tn}* animals. In addition, *Nrg1^{Tn}* rats, regardless of sex, showed higher basal CORT levels than WT rats ($p < 0.03$). At the peak time point (B), both WT (** $p < 0.01$) and *Nrg1^{Tn}* (** $p < 0.001$) females exhibited greater CORT secretion than males. At the recovery time point (C) WT female CORT levels were significantly greater than WT male CORT (* $p < 0.02$) whereas *Nrg1^{Tn}* males and females were indistinguishable. *Nrg1^{Tn}* rats, regardless of sex, show lower recovery CORT levels than WT rats ($p < 0.04$). Bars represent mean $\pm$ SEM.

Figure 3.

Female open field testing and habituation. Overall, *Nrg1^{Tn}* females (A) spent less time mobile (* $p < 0.02$), (B) made fewer line crossings (* $p < 0.03$) and (C) traveled less distance (* $p < 0.05$) than WT rats. Both WT and *Nrg1^{Tn}* females habituated to the open field across exposures, demonstrating decreased locomotor activity from exposure 1 to exposure 2. However, on exposure 1, *Nrg1^{Tn}* females exhibited enhanced habituation, as measured by percent decrease, in (D) time mobile (** $p < 0.01$), (E) line crossings (** $p < 0.001$) and (F) distance traveled (** $p < 0.001$). Bars represent mean $\pm$ SEM.

Figure 4.

Sex differences in locomotor activity. No sex difference between WT males and females was detected on the locomotor index, a combined measure of time mobile, line crossings and distance traveled. However, a sex difference was detected in *Nrg1^{Tn}* rats, with males exhibiting greater locomotor activity compare to females (**p<0.001). Bars represent mean ± SEM.

Figure 5.

Acoustic startle response and prepulse inhibition. On baseline ASR (A) an effect of sex ($p < 0.02$) but not genotype was found, with males showing greater startle amplitudes than females. On ASR habituation (B), measured by percent decrease in startle amplitude, an effect of genotype ($*p < 0.02$) was found, wherein *Nrg1^{Tn}* rats exhibited greater habituation to acoustic startle stimuli than WT rats. (C) PPI trials are presented by group. Within each group, bars represent trials of increasing prepulse intensity (from left to right: 3dB, 6dB, 12dB, 15dB). *Nrg1^{Tn}* females showed an overall reduction in PPI compared to WT females ($*p < 0.03$) while WT and *Nrg1^{Tn}* males were indistinguishable. Bars represent mean $\pm$ SEM.

Table 1

Female pubertal onset and adult reproductive function.

Genotype	Day of Vaginal Opening	Cycle Length (days)	Days to First Litter	Number Born	Percent Survival	Percent Male
WT	38.5 ± 0.38	5.3 ± 0.19	29.9 ± 0.97	7.7 ± 0.80	81.6 ± 9.8	37.7 ± 6.3
<i>Nrg1^{fl/y}</i>	39.0 ± 0.33	5.5 ± 0.20	27.1 ± 0.77	9.3 ± 0.93	76.5 ± 8.3	44.5 ± 8.0

mean ± SEM

Table 2

Glucocorticoid (GR) and mineralocorticoid (MR) receptor protein expression in females.

Genotype	Pituitary		PVN		HPC			AMG		
	GR		GR		GR	MR	MR/GR	GR	MR	MR/GR
WT	1.37 ± 0.08		2.11 ± 0.33		1.17 ± 0.1	0.49 ± 0.07	0.49 ± 0.11	0.89 ± 0.16	0.84 ± 0.03	1.16 ± 0.18
<i>Nrg1^{fln}</i>	0.97 ± 0.07**		2.07 ± .022		1.12 ± 0.07	0.45 ± 0.06	0.40 ± 0.04	1.14 ± 0.10	0.81 ± 0.05	0.72 ± 0.05#

mean ± SEM

** compared to WT in same region (p<0.01)

compared to WT in same region (p≤0.07)

Table 3

Summary of sex differences.

Measure	WT		<i>Nrg1^{fl/y}</i>		Sex Difference
	♂	♀	♂	♀	
CORT					
Basal	<	-	-	-	Eliminated
Peak	<	<	<	<	Preserved
Recovery	<	-	-	-	Eliminated
Locomotor	-	>	>	>	Emerges
ASR	>	>	>	>	Preserved
PPI	-	-	-	-	Preserved