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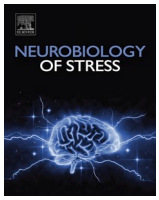

Imbalanced glucocorticoid and mineralocorticoid stress hormone receptor function has sex-dependent and independent regulatory effects in the mouse hippocampus

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ABSTRACT

Many stress-related neuropsychiatric disorders display pronounced sex differences in their frequency and clinical symptoms. Glucocorticoids are primary stress hormones that have been implicated in the development of these disorders but whether they contribute to the observed sex bias is poorly understood. Glucocorticoids signal through two closely related nuclear receptors, the glucocorticoid (GR) and mineralocorticoid receptor (MR). To elucidate the sex-specific and independent actions of glucocorticoids in the hippocampus, we developed knockout mice lacking hippocampal GR, MR, or both GR and MR. Mice deficient in hippocampal MR or both GR and MR showed an altered molecular phenotype of CA2 neurons and reduced anxiety-like behavior in both sexes, but altered stress adaptation behavior only in females and enhanced fear-motivated cue learning only in males. All three knockout mouse models displayed reduced sociability but only in male mice. Male and female mice deficient in both hippocampal GR and MR exhibited extensive neurodegeneration in the dentate gyrus. Global transcriptomic analysis revealed a marked expansion in the number of dysregulated genes in the hippocampus of female knockout mice compared to their male counterparts; however, the overall patterns of gene dysregulation were remarkably similar in both sexes. Within and across sex comparisons identified key GR and MR target genes and associated signaling pathways underlying the knockout phenotypes. These findings define major sex-dependent and independent effects of GR/MR imbalances on gene expression and functional profiles in the hippocampus and inform new strategies for treating men and women with stress-related neuropsychiatric disorders.

1. Introduction

Many stress-related neuropsychiatric disorders display pronounced sex differences in their frequency and severity of symptoms (Bangasser and Valentino, 2014). Depression, anxiety, and posttraumatic stress disorder (PTSD) are more prevalent in women (Kessler, 2003; Sheikh et al., 2002; Tolin and Foa, 2006). In contrast, schizophrenia and substance abuse related disorders are more frequently observed in men (Grant et al., 2004; Thomas et al., 2010). Elucidating the molecular mechanisms underlying these sex biases is critical for the development of more effective and personalized treatments to combat these debilitating conditions.

Glucocorticoids are primary stress hormones that are secreted by the adrenal glands in response to circadian and stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation (Oakley and Cidlowski, 2013). These hormones regulate numerous biological processes involved in stress adaptation and maintenance of homeostasis, and their ability to suppress the immune system has made synthetic glucocorticoids one of the most prescribed drug classes in the world today (Cain and Cidlowski, 2017; Rhen and Cidlowski, 2005). The actions of glucocorticoids are mediated by two highly homologous nuclear receptors, the glucocorticoid (GR) and mineralocorticoid receptor (MR). MR binds glucocorticoids (cortisol in humans; corticosterone in rodents) with a greater affinity than GR (Reul and de Kloet, 1985). Consequently, MR is occupied predominantly by glucocorticoids under basal conditions when HPA activity is low, whereas GR becomes occupied progressively

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as hormone levels rise during the circadian peak and in response to stress (De Kloet et al., 1998). Both hormone-bound GR and MR regulate the expression of numerous target genes by binding nearly identical DNA glucocorticoid response elements (GREs).

In the brain, glucocorticoids act on the hippocampus to regulate cognition, mood, and HPA axis activity. A distinguishing feature of the hippocampus is that it expresses high levels of GR and MR suggesting it may be especially sensitive to aberrant levels of stress and disturbances in the balance of these two receptors (Seckl et al., 1991; Watzka et al., 2000). Indeed, imbalances in hippocampal GR and MR signaling have been associated with stress-related learning and memory impairments, depression, anxiety, and PTSD (de Kloet et al., 2005; Herman et al., 2012; Laryea et al., 2015; Yehuda, 2001). However, the molecular mechanisms by which perturbations in these receptors regulate vulnerability or resistance to these disorders remains poorly understood. Moreover, few studies have examined whether GR/MR imbalances also contribute to the pronounced sex differences in these disorders.

We recently developed mice with conditional knockout of GR, MR, or both GR and MR in the hippocampus (Oakley et al., 2021). Our initial studies on these mice were performed on males and revealed distinct pathologies and gene expression profiles that were associated with individual or combinatorial actions of GR and MR. The current study was undertaken to compare male and female single and double knockout mice in order to elucidate the direct sex-dependent and independent actions of GR and MR in the hippocampus. We hypothesized that manipulating the hippocampal GR/MR balance would lead to sex-dependent and independent genetic signatures and functional phenotypes.

2. Material and methods

2.1. Generation of $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice

Mice with a floxed GR locus ($GR^{fl/fl}$), floxed MR locus ($MR^{fl/fl}$), and with both alleles floxed ($GR^{fl/fl}MR^{fl/fl}$) were each crossed with mice expressing Cre recombinase under the direction of the empty spiracles homeobox 1 (*Emx1*) locus (The Jackson Laboratory, 005628). The resulting $GR^{fl/fl}Emx1^{Cre/+}$ ($GR^{Emx1-cre}$), $MR^{fl/fl}Emx1^{Cre/+}$ ($MR^{Emx1-cre}$), and $GR^{fl/fl}MR^{fl/fl}Emx1^{Cre/+}$ ($GRMR^{Emx1-cre}$) mice have been previously described (Oakley et al., 2021). Cre negative $GR^{fl/fl}Emx1^{+/+}$ (GRflox), $MR^{fl/fl}Emx1^{+/+}$ (MRflox), and $GR^{fl/fl}MR^{fl/fl}Emx1^{+/+}$ (dflox) mice served as controls. All mice were on a C57BL/6NJ background. Genotypes were determined using real time PCR with specific probes designed for each gene (Transnetyx). Mice were housed on a 12h:12h light/dark cycle with access to food and water *ad libitum*. Experiments on mice were approved and performed according to the guidelines of the Animal Care and Use Committee (NIEHS) and the Institutional Animal Care and Use Committee (UNC) and were in accordance with the NIH guide for care and use of laboratory animals. All efforts were made to minimize animal suffering, to reduce the number of mice used, and to use alternatives to in vivo techniques, if available.

2.2. Real-time PCR

The mouse brain was removed from the skull and bisected along the interhemispheric fissure. From the medial surface of each brain half, the diencephalon and brain stem were removed to expose the hippocampus. The hippocampal formation was “rolled” out being careful to remove any attached cortex, incubated in RNA stabilization reagent (Qiagen), and then frozen at −80 °C. The hippocampus from both hemispheres of each mouse brain was combined together, and total RNA was isolated using RNeasy mini kit (Qiagen). RNA was analyzed with the CFX96 RTPCR (BioRad) sequence detection system using primer sets for RTPCR that were purchased from ThermoFisher Scientific. The relative expression values for each gene were calculated using the double delta Ct analysis method and the housekeeping gene peptidylprolyl isomerase B (*Ppib*).

2.3. Immunoblotting

For each mouse, the hippocampus was removed as described above from both hemispheres of the brain, combined together, and then lysed in sodium dodecyl sulfate (SDS) sample buffer. Equivalent amounts of protein were electrophoresed, transferred to nitrocellulose membranes, and analyzed and quantitated using the Odyssey Infrared Imaging System (LI-COR Biosciences). Primary antibodies were to GR (Cell Signaling Technology, #3660, RRID:AB_11179215), MR (clone 6G1 generously provided by Dr. Celso E. Gomez-Sanchez and available from Millipore, #MABS496, RRID:AB_2811270) (Gomez-Sanchez et al., 2006), and actin (Millipore, #MAB1501, RRID:AB_2223041). Secondary antibodies utilized were goat anti-rabbit Alexa Fluor 680-conjugated (Life Technologies, #A-21109, RRID:AB_2535758) and goat anti-mouse IRDye800-conjugated (LI-COR Biosciences, #926–32210, RRID: AB_621842).

2.4. Corticosterone measurements

Mandibular bleeds were performed as previously described on adult female mice that did not undergo behavioral testing (Oakley et al., 2021). The bleeds occurred in the morning (between 8:30 and 9:30 a.m.), in the evening (between 7:00 and 8:30 p.m.), and in the morning (between 7:00 and 9:30 a.m.) immediately following restraint stress. The different bleeds were separated by at least one week. Mice were placed in ventilated holders (Kent Scientific) for 30 min for the restraint stress experiment. In an effort to minimize variability, the venipuncture was performed by the same individual on all mice and occurred within approximately 1–2 min of touching the cage for the morning and evening measurements and immediately after removing the mouse from the ventilated holder for the restraint stress measurement. Blood was collected using Goldenrod lancets #5 (Fisher Scientific) into serum separator tubes (BD Microtainer, Fisher Scientific), and the serum was separated by centrifugation. Serum corticosterone levels were measured using an Enzyme Immunoassay Kit (Arbor Assays, #K014-H1, Ann Arbor, MI).

2.5. Histological analysis

Mice were perfused transcardially with saline followed by 4% paraformaldehyde. Brains were removed, fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose in PBS, and then frozen in embedding medium (OCT, Sakura Finetek, Torrance, CA). A cryostat was used to prepare 40-μm coronal sections of the brain, and these free-floating sections were stored at 4 °C in phosphate buffered saline (PBS) supplemented with sodium azide (0.01%). Nissl stains were performed following standard protocols on 40-μm sections mounted on gelatin subbed slides (Southern Biotech). The area of the dentate gyrus granule cell layer at approximate Bregma level −1.7 mm was determined from Nissl-stained sections using NIH Image J (FIJI) software and represents the average from both hemispheres. For immunofluorescence studies, 40-μm free floating sections were processed for antigen retrieval by boiling in a citrate-based antigen unmasking solution (Vector Laboratories) for 5 min. Primary antibodies were rabbit anti-GR (Cell Signaling Technology, #3660, RRID:AB_11179215), mouse anti-MR (clone 6G1 generously provided by Dr. Celso E. Gomez-Sanchez and available from Millipore, #MABS496, RRID:AB_2811270) (Gomez-Sanchez et al., 2006), rabbit anti-PCP4 (Sigma-Aldrich, #HPA005792, RRID: AB_1855086), and mouse anti-RGS14 (Antibodies Incorporated, #75–170, RRID:AB_2179931). Secondary antibodies were goat anti-mouse Alexa Fluor 488 (Life Technologies, #A-11001, RRID: AB_2534069), goat anti-rabbit Alexa Fluor 594 (Life Technologies, #A-11012, RRID:AB_141359), goat anti-mouse Alexa Fluor 594 (Life Technologies, #A-11005, RRID:AB_141372), and goat anti-rabbit Alexa

Fluor 488 (Life Technologies, #A-11034, RRID:AB_2576217). Sections were mounted using VECTASHIELD antifade mounting medium with DAPI (Vector Laboratories). Nissl images were captured using an automated Zeiss epifluorescent microscope. Immunofluorescent images were acquired using a Zeiss LSM 780 confocal microscope. The 488 and 594 laser settings were identical for flox control and knockout sections within experiments, and Adobe Photoshop color level adjustments were applied equally to all images within experiments. For some experiments, brains were embedded in paraffin and 8-μm coronal sections were collected using a vibratome and mounted on slides. Hematoxylin and eosin staining was performed on paraffin sections according to standard protocols.

2.6. Microarray analysis

Global gene expression analysis was performed on RNA isolated from the hippocampus (from both hemispheres combined together for each mouse brain) from ~2.5-month old male and female dflox control, $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice (n = 4 mice per genotype). The Agilent Whole Mouse Genome oligo arrays (014868) (Agilent Technologies) were used following the Agilent 1-color microarray-based gene expression analysis protocol as described previously (Oakley et al., 2019). One male $GRMR^{Emx1-cre}$ sample was identified as an outlier from the principal component analysis and removed. Separate analysis of the male data has been previously published (Oakley et al., 2021). For this study, the combined male and female data were analyzed together in order to elucidate the sex-dependent and independent gene changes. To determine differentially expressed probes by sex (female flox control vs male flox control) and genotype (male $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, or $GRMR^{Emx1-cre}$ vs male flox control; female $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, or $GRMR^{Emx1-cre}$ vs female flox control), an ANOVA with multiple test correction (FDR q-value <0.01) was performed across the combined male and female data using Partek Genomics Suite software, version 6.6 (Partek). The statistically significant probes were analyzed by Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) for enrichment of neurotransmitter and other nervous system signaling pathways, diseases and disorders, and molecular and cellular functions. Gene enrichment P values (p-value <0.05) were determined by IPA using Fisher's exact test. Potential GR and MR target genes were identified using the IPA Upstream Regulator analysis function and the following significant upstream regulators: aldosterone, corticosterone, dexamethasone, glucocorticoid, mineralocorticoid, Nr3c1, and Nr3c2. Microarray data have been deposited in Gene Expression Omnibus with the accession number GSE240873.

2.7. Behavioral assays

Behavioral testing was carried out by experimenters blinded to genotype, using subject lists and home-cage cards without genotype information. Subject order for testing was approximately balanced across genotype by an experimenter not involved in conducting the behavior assays, to control for time of testing (i.e. early, mid-way, or later during each test session). Behavioral assays were performed during the light phase of the light/dark cycle using published methods (Goulding et al., 2019). Control male mice were comprised of 9 GRflox, 7–8 MRflox, and 10 dflox mice. One male MRflox mouse was lost over the course of the behavioral studies for unknown causes. Control female mice were comprised of 9 GRflox, 9 MRflox, and 8 dflox mice. Knockout male mice were comprised of 12 $GR^{Emx1-cre}$, 11–12 $MR^{Emx1-cre}$, and 9–11 $GRMR^{Emx1-cre}$ mice. Over the course of the behavioral studies, one male $MR^{Emx1-cre}$ and one male $GRMR^{Emx1-cre}$ mouse were lost for unknown causes and one male $GRMR^{Emx1-cre}$ mouse was lost due to fight wounds. Knockout female mice were comprised of 12 $GR^{Emx1-cre}$, 11 $MR^{Emx1-cre}$, and 12 $GRMR^{Emx1-cre}$ mice. The same group of male mice and the same group of female mice underwent all behavioral tests and followed the same testing regimen, with the behavioral study on the female mice taking place approximately 2 years after the male mice. Mice were approximately 7 weeks in age at the beginning of the regimen, with assays carried out in the following order: elevated plus maze (~7 weeks of age), three chamber test for sociability (~9 weeks of age), conditioned fear test (~12 weeks of age), and the forced swim test (~15 weeks of age).

2.8. Elevated plus maze test

Mice were evaluated for anxiety-like behavior during a 5-min test in the elevated plus maze, which had two walled arms (the closed arms, 20 cm in height) and two open arms. The maze was elevated 50 cm from the floor, and the arms were 30 cm long. Mice were placed on the center section and allowed to freely explore the maze. Measures were taken of the time on, and the number of entries into, the open and closed arms.

2.9. Three-chamber choice test for sociability

The choice test consisted of two 10-min phases: a habituation period, followed by a test for sociability. During the sociability phase, mice were given a choice between spending time in proximity to an unfamiliar mouse (the "stranger"), versus a non-social novel object (an empty cage). The testing apparatus was a rectangular, 3-chambered box fabricated from clear Plexiglas. Dividing walls had doorways allowing access into each chamber. At the start of the test, the mouse was placed in the middle chamber and allowed to explore with the doorways into the two side chambers open. After the habituation period, the test mouse was enclosed in the center compartment of the social test box, and an unfamiliar sex-matched C57BL/6J adult mouse (the stranger) was placed in one of the side chambers. The stranger mouse was enclosed in a small Plexiglas cage drilled with holes. An identical empty Plexiglas cage was placed in the opposite side of the chamber. Following placement of the stranger and the empty cage, the doors were re-opened, and the subject was allowed to explore the entire social test box for 10-min. An automated image tracking system (Noldus Ethovision) provided measures of time spent in each side, time spent in 5-cm proximity to each cage, and numbers of entries into each side of the social test box.

2.10. Conditioned fear test

At approximately 12 weeks of age, mice were evaluated for learning and memory in a conditioned fear test (Near-Infrared image tracking system, MED Associates, Burlington, VT) as previously described (Oakley et al., 2021). The procedure had the following phases: training on Day 1, context-dependent learning test on Day 2, and cue-dependent learning test on Day 3. Two weeks later, mice were given second tests for retention of contextual (Day 16) and cue (Day 17) learning. During the Day 1 training, mice were exposed to 3 shock-tone pairings with 80 s between each pairing. Each pairing consisted of a 30-sec tone (80 dB) that co-terminated with a 2-sec scrambled foot shock (0.4 mA). On Day 2 and 16, mice were placed back into the original conditioning chamber and levels of freezing were determined across a 5-min contextual learning test. On Day 3 and 17, mice were placed in a modified conditioning chamber for a cue learning test. After 2 min, the acoustic stimulus (an 80 dB tone) was presented for a 3-min period. Levels of freezing before and during the stimulus were determined across a 5-min test. Data for male mice has been previously published (Oakley et al., 2021) and is presented again for comparative purposes with female data.

2.11. Forced swim test

Mice approximately 15 weeks old were evaluated for stress adaptation behavior in the forced swim test. In brief, mice were placed in a clear beaker filled with 12–15 cm of clean tap water (23–26 °C) for a 6-min test. Measures of time spent immobile and swimming distance were taken by an automated image tracking system (Noldus Ethovision)

during the last 4 min of the test.

2.12. Statistics

One-way ANOVA with Tukey's multiple comparisons test was used to evaluate statistical significance (defined as p-value <0.05). The Mantel-Cox log-rank test was used for survival curves. Behavioral data were first analyzed using 2-way ANOVAs or repeated measures ANOVAs, with the factors genotype and sex. A limitation for the interpretation of these analyses was that the male and female groups were evaluated at different times in the behavioral assays. Data from males and females were then analyzed separately using one-way ANOVA or repeated measures ANOVAs to determine effects of genotype within each sex. Fisher's protected least-significant difference (PLSD) tests were used for comparing group means only when a significant F value for genotype was determined in the ANOVA. For dentate gyrus granule cell layer area, data were first analyzed using 2-way ANOVAs with factors genotype and sex. Male and female data were then analyzed separately using an unpaired two-tailed *t*-test. The statistical analyses were performed using Statview (SAS, Cary, NC) and GraphPad Prism software (version 9.5.0; Dotmatics, Boston, MA).

3. Results

3.1. Characterization of female $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice

We previously developed and characterized mice with conditional knockout of GR ($GR^{Emx1-cre}$), MR ($MR^{Emx1-cre}$), or both GR and MR in the hippocampus ($GRMR^{Emx1-cre}$) (Oakley et al., 2021). Our initial studies on these mice, however, were performed only on males. To examine the sex-dependent and independent actions of glucocorticoids in the hippocampus, we have now performed studies on female single and double knockout mice and compared the results to their male counterparts. We first evaluated the expression of GR and MR in female flox control and knockout mice (Fig. 1A and B). The $GR^{Emx1-cre}$ mice showed reduced levels of hippocampal GR mRNA and protein and no change in the levels of MR. The $MR^{Emx1-cre}$ mice showed reduced levels of hippocampal MR mRNA and protein and a significant increase in GR mRNA and protein. The $GRMR^{Emx1-cre}$ mice showed reduced expression of both GR and MR in the hippocampus. These results demonstrate that the female single and double knockout mice have alterations in hippocampal GR and MR expression levels that are similar to those previously reported for their male counterparts (Oakley et al., 2021). Our finding that GR is upregulated in the $MR^{Emx1-cre}$ hippocampus in both male and female mice is important because it suggests that MR normally functions in a sex-independent manner to limit GR expression in the hippocampus. Moreover, it raises the possibility that aberrant GR signaling, rather than deficient MR signaling, contributes to observed phenotypes in the $MR^{Emx1-cre}$ mice. A key feature of the $GRMR^{Emx1-cre}$ mouse model is that by eliminating the expression of both GR and MR it can distinguish between these two scenarios and reveal the molecular culprit behind the phenotypic change in the $MR^{Emx1-cre}$ mice.

The female $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice were born at the expected Mendelian frequency and survived normally through 12 months of age (Fig. 1C). Female $GR^{Emx1-cre}$ mice showed normal growth with no alteration in body weight throughout the 12-month study (Fig. 1D), whereas body weight reductions were measured for 3-month old female $MR^{Emx1-cre}$ mice (9.7% decrease compared to MRflox control mice) and for 12-month old female $GRMR^{Emx1-cre}$ mice (18.2% decrease compared to dflox control mice) (Fig. 1D). Similar survival and body weight changes were previously reported for male $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice (Oakley et al., 2021). The loss of GR, MR, or both receptors in the hippocampus of female mice did not lead to any significant alterations in circadian or stress-induced levels of corticosterone which is consistent with our findings from the male knockout mice (Fig. 1E) (Oakley et al., 2021). Collectively, these data suggest that knockout of GR, MR, or both receptors in the male and female hippocampus does not impact survival and HPA axis activity but does lead to mild growth defects that occur in a sex independent manner in the $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice.

3.2. Hippocampal MR signaling maintains molecular identity of CA2 neurons in a sex independent manner

Immunostaining was performed to evaluate the expression level and distribution of GR and MR in the hippocampus of female flox control and knockout mice. Results show that GR and MR are both expressed in CA1, CA2, and CA3 pyramidal neurons and in dentate gyrus granule neurons of the flox control hippocampus; however, the level of receptor expression differed across these regions (Fig. 2A). The rank order of GR expression was CA1 $>$ dentate gyrus $>>$ CA2 $\approx$ CA3, whereas the rank order of MR expression was CA2 $>$ CA1 $\approx$ CA3 $\approx$ dentate gyrus. In all regions of the hippocampus, GR and MR were found to colocalize in the same neurons. For female $GR^{Emx1-cre}$ mice, GR expression was abolished in all hippocampal regions and MR expression was unaffected (Fig. 2A). For female $MR^{Emx1-cre}$ mice, MR expression was abolished in all hippocampal regions and GR expression was upregulated particularly in the CA2 and CA3 neurons that normally express low levels of GR (Fig. 2A). Higher magnification images clearly depict the differential expression of GR and MR at the CA1/CA2 boundary in flox control mice and the marked upregulation of GR in CA2 and CA3 neurons in the $MR^{Emx1-cre}$ hippocampus (Fig. 2B). This finding is consistent with the increase in GR mRNA and protein that we observed in whole hippocampal lysates from female $MR^{Emx1-cre}$ mice (Fig. 1A and B). For the female $GRMR^{Emx1-cre}$ hippocampus, both GR and MR expression were abolished in all regions of the hippocampus. The unique GR and MR distribution patterns observed in the female flox control hippocampus and the marked upregulation of GR in CA2 and CA3 neurons in the $MR^{Emx1-cre}$ mice are similar to our previously published findings in male mice indicating that they occur in a sex independent manner (Oakley et al., 2021).

Since MR deficiency resulted in the aberrant upregulation of GR in CA2 neurons of female $MR^{Emx1-cre}$ mice (Fig. 2A and B), we examined these neurons further in our knockout mice for alterations in the classic CA2-specific markers, Purkinje cell protein 4 (PCP4) and regulator of G protein signaling 14 (RGS14) (Fig. 2C). Immunostaining revealed robust expression of PCP4 and RGS14 in CA2 neurons in female flox control and $GR^{Emx1-cre}$ mice. In marked contrast, PCP4 and RGS14 expression were abolished in the CA2 neurons in the female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice. The double knockout mice lack both GR and MR but show the same alteration in CA2 markers as the $MR^{Emx1-cre}$ mice which have elevated levels of GR; therefore, we conclude that the molecular culprit underlying the loss of PCP4 and RGS14 expression in the $MR^{Emx1-cre}$ hippocampus is the deficiency in MR signaling. Similar reductions in PCP4 and RGS14 expression were observed previously in CA2 neurons in the male $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice (Oakley et al., 2021). These findings demonstrate that hippocampal MR signaling is required for maintaining the molecular identity of CA2 neurons, and this occurs in a sex independent manner.

3.3. Hippocampal GR and MR signaling cooperate to prevent neurodegeneration in the dentate gyrus in a sex independent manner

In our previous study performed on males (Oakley et al., 2021), we observed extensive neurodegeneration in the dentate gyrus of the $GRMR^{Emx1-cre}$ mice but not in their single knockout counterparts. To determine whether this neuronal loss also occurred in female mice lacking both GR and MR, we examined the morphology of the hippocampus from 3-month old $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice using Nissl staining (Fig. 3A). The female $GR^{Emx1-cre}$ and $MR^{Emx1-cre}$ hippocampi showed a normal density of granule neurons in the dentate gyrus. In marked contrast, neuronal loss was evident along

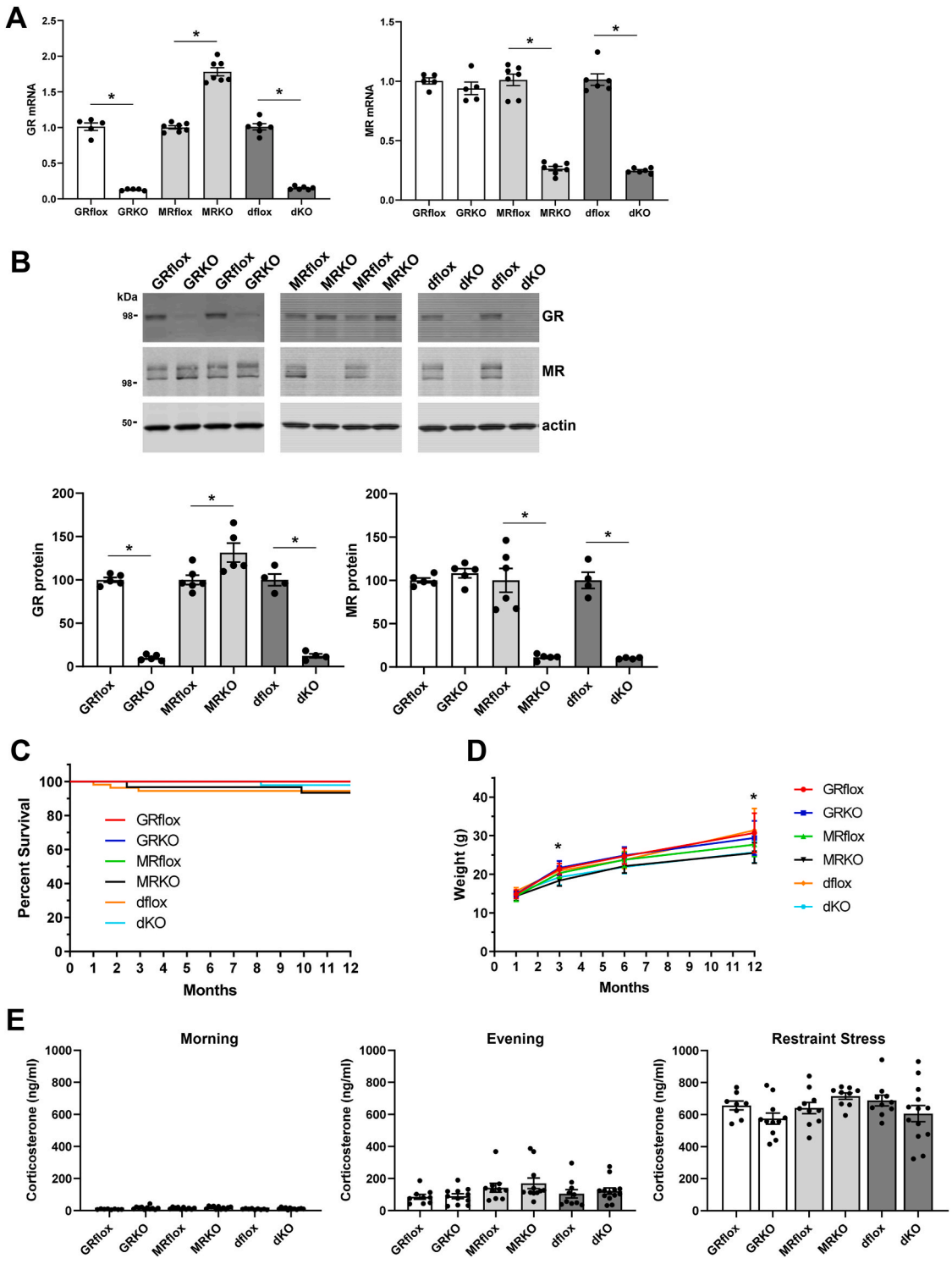

Fig. 1. Conditional knockout of GR, MR, or both GR and MR in the hippocampus of female mice. (**A**) RTPCR of GR and MR mRNA from the hippocampus of 2- to 4-month old female flox controls and $GR^{Emx1-cre}$ (GRKO), $MR^{Emx1-cre}$ (MRKO), and $GRMR^{Emx1-cre}$ (dKO) mice. Data are mean ± SEM (n = 5–7 mice per group). A one-way ANOVA followed by Tukey post-hoc analysis was performed to determine significance. *P < 0.05 for GRKO compared to GRflox, MRKO compared to MRflox, and dKO compared to dflox. (**B**) Representative immunoblots and quantitation of GR and MR protein levels from the hippocampus of 4- to 6-month old female mice. Data are mean ± SEM (n = 4–6 mice per group). A one-way ANOVA followed by Tukey post-hoc analysis was performed to determine significance. *P < 0.05 for GRKO compared to GRflox, MRKO compared to MRflox, and dKO compared to dflox (**C**) Survival curves for female GRflox (n = 38), $GR^{Emx1-cre}$ (n = 35), MRflox (n = 32), $MR^{Emx1-cre}$ (n = 30), dflox (n = 54), and $GRMR^{Emx1-cre}$ (n = 46) mice. Mantel-Cox log-rank test revealed no significant differences among genotypes. (**D**) Body weights were measured for female flox control and knockout mice at 1, 3, 6, and 12 months of age. Data are mean ± SEM (n = 11–40 mice per group). A one-way ANOVA followed by Tukey post-hoc analysis was performed to determine significance. *P < 0.05 for MRKO compared to MRflox and dKO compared to dflox. (**E**) Corticosterone levels in the morning, evening, and in the morning immediately following 30 min of restraint stress in adult female mice. Data are mean ± SEM (n = 8–13 mice per group). A one-way ANOVA revealed no significant differences among genotypes.

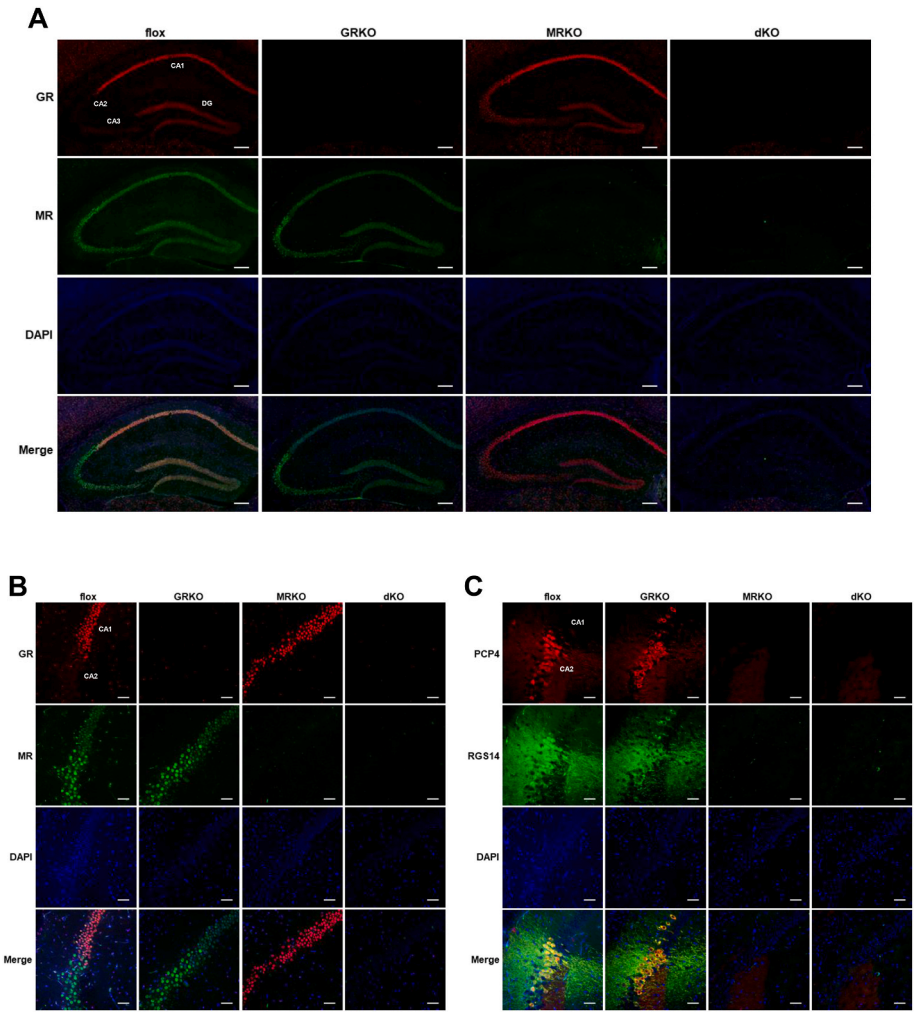

Fig. 2. Distribution of GR, MR, and the CA2 neuronal markers PCP4 and RGS14 in the hippocampus of female control and knockout mice. Immunofluorescence staining was performed on hippocampal sections from ~3-month old female flox control and knockout mice. (**A**) Representative images of the distribution of GR and MR in the whole hippocampus (n = 3–4 mice per genotype). Scale bars, 200 μm. (**B**) Representative images of the distribution of GR and MR at the CA1/CA2 boundary (n = 3–4 mice per genotype). Scale bars, 50 μm. (**C**) Representative images of the distribution of the CA2 neuronal markers PCP4 and RGS14 in flox control and knockout mice (n = 3–4 mice per genotype). Scale bars, 50 μm.

the dorsal blade of the dentate gyrus in the female double knockout mice (Fig. 3A, see arrows). At this 3-month time point, the magnitude of neuronal loss in the female GRMR$^{Emx1-cre}$ dentate gyrus was variable, ranging from minor to moderate to extensive (Fig. 3A and B). Similar variability in the degree of neuronal loss was also observed in 3-month old male GRMR$^{Emx1-cre}$ mice (Fig. 3B) (Oakley et al., 2021). To estimate the degree of neuronal loss at this time point, we measured the area of the dentate gyrus granule cell layer from the Nissl-stained sections (Fig. 3C). Overall two-way ANOVAs revealed a main effect of genotype [$F(1,10) = 19.81$, $p < 0.0012$], with no significant interactions between genotype and sex. Post-hoc testing indicated that both male and female GRMR$^{Emx1-cre}$ mice exhibited significant neuronal loss of approximately 30% in the dentate gyrus by 3 months of age. By 4 months of age, extensive neurodegeneration in the dentate gyrus was consistently observed in the majority of female and male GRMR$^{Emx1-cre}$ mice (Fig. 3D) (Oakley et al., 2021). To determine if the neuronal loss in the female dentate gyrus persisted and remained specific to the double knockout mice, we performed Nissl staining on hippocampi from 12-month old female GR$^{Emx1-cre}$, MR$^{Emx1-cre}$, and GRMR$^{Emx1-cre}$ mice (Fig. 3E). As previously observed for the males (Oakley et al., 2021), the female GR$^{Emx1-cre}$ and MR$^{Emx1-cre}$ mice exhibited a normal density of granule neurons at this 12 month time point whereas prominent neurodegeneration continued to be observed in the dentate gyrus of the female GRMR$^{Emx1-cre}$ hippocampus (Fig. 3E, arrows). These data suggest that hippocampal GR and MR work together in a sex independent manner to preserve granule neurons in the dentate gyrus.

3.4. Hippocampal GR and MR signaling regulate behavior in both a sex dependent and independent manner

Since alterations in hippocampal glucocorticoid signaling have been implicated in a variety of neuropsychiatric disorders that display sex differences in disease susceptibility (Bangasser and Valentino, 2014; de Kloet et al., 2005; Herman et al., 2012; Laryea et al., 2015; Yehuda, 2001), we evaluated male and female GR$^{Emx1-cre}$, MR$^{Emx1-cre}$, and GRMR$^{Emx1-cre}$ mice for behavioral changes relevant to anxiety, stress adaptation, sociability, and cognitive function. Anxiety-like behavior was evaluated using the elevated plus maze test (Fig. 4A). Mice were placed in the center section of the maze and allowed to freely explore for 5 min. The time spent on the open arms and the number of entries into the open arms are indices of anxiety-like behavior. Overall two-way ANOVAs revealed a main effect of genotype on percent open arm time [$F(3,115) = 18.41$, $p < 0.0001$] and percent open arm entries [$F(3,115) = 19.41$, $p < 0.0001$], with no significant interactions between genotype and sex. Post-hoc testing indicated that both male and female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice exhibited reduced anxiety-like

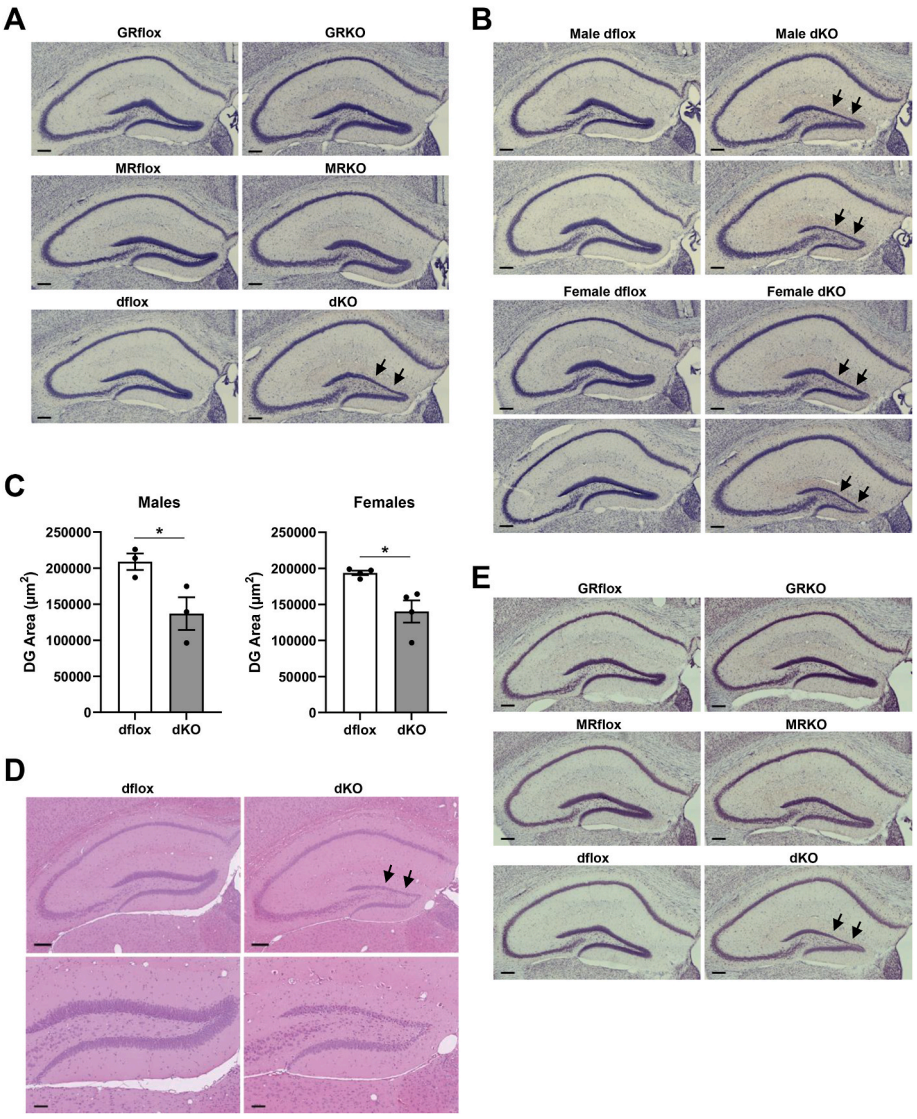

Fig. 3. Female GRMR$^{Emx1\text{-cre}}$ mice exhibit neurodegeneration in the dentate gyrus. (**A**) Representative Nissl stains of hippocampus from ~3-month old female flox control and knockout mice (n = 3–4 mice per genotype). Scale bars, 200 μm. (**B**) Nissl stains of hippocampus from ~3-month old male and female mice showing variable levels of neurodegeneration. Scale bars, 200 μm. (**C**) The area of the dentate gyrus (DG) granule cell layer at approximate Bregma level −1.7 mm was determined from Nissl-stained sections from ~3-month old male and female mice. Data are mean ± SEM (n = 3–4 mice per genotype). Students t-test was performed to determine significance. *P < 0.05 for dKO compared to flox control. (**D**) Representative hematoxylin and eosin stains of hippocampus from ~4-month old female mice (n = 3–4 mice per genotype). Lower panels show higher magnification images of dentate gyrus in upper panels. Scale bars, 200 μm (upper images) and 80 μm (lower images). (**E**) Representative Nissl stains of hippocampus from ~12-month old female mice (n = 4–5 mice per genotype). Scale bars, 200 μm. Arrows show granule neuron loss along dorsal blade of dentate gyrus.

behavior as they spent more percent time on the open arms and made more entries into the open arms compared to flox control mice. Since the double knockout mice show the same low-anxiety phenotype as the MR$^{Emx1-\text{cre}}$ mice which have elevated levels of GR, we conclude that the molecular culprit underlying the reduced anxiety-like behavior is the deficiency in MR signaling. The total number of arm entries was also measured to provide an index of general activity (Fig. 4A). Overall two-way ANOVAs revealed a main effect of genotype [$F(3,115) = 8.72$, $p < 0.0001$] and a main effect of sex [$F(1,115) = 7.39$, $p = 0.0076$] on total arm entries but no significant interaction between genotype and sex. Post-hoc testing indicated that both male and female MR$^{Emx1-\text{cre}}$ and GRMR$^{Emx1-\text{cre}}$ mice made more total arm entries than flox control mice (trend for male GRMR$^{Emx1-\text{cre}}$ mice, $P = 0.096$) which is consistent with previous reports associating MR deficiency in the brain with hyper-reactivity to a novel environment (McCann et al., 2021). Taken together, these data suggest that hippocampal MR signaling acts in a sex independent manner to regulate the anxiogenic response in male and female mice.

We evaluated stress adaptation behavior in male and female knockout mice using the forced swim test (Fig. 4B). Mice were placed in a beaker of water for 6 min, and measures of time spent immobile (passive coping) and swimming distance (active coping) were taken during the last 4 min. In this test, mice initially swim to escape and then progressively assume an immobile position, and this switch from active to passive coping provides an index of stress adaptation (Molendijk and de Kloet, 2015; Molendijk and de Kloet, 2022). Overall two-way ANOVAs revealed a significant genotype and sex interaction on immobility time [$F(3,111) = 2.91$, $p = 0.038$]. For swimming distance, a main effect of sex [$F(1,111) = 4.24$, $p = 0.0419$] and trend for a significant interaction between genotype and sex [$F(3,111) = 2.57$, $p = 0.0581$] were detected. Post-hoc testing revealed no genotype differences for immobile floating or swimming activity in male knockout mice. In

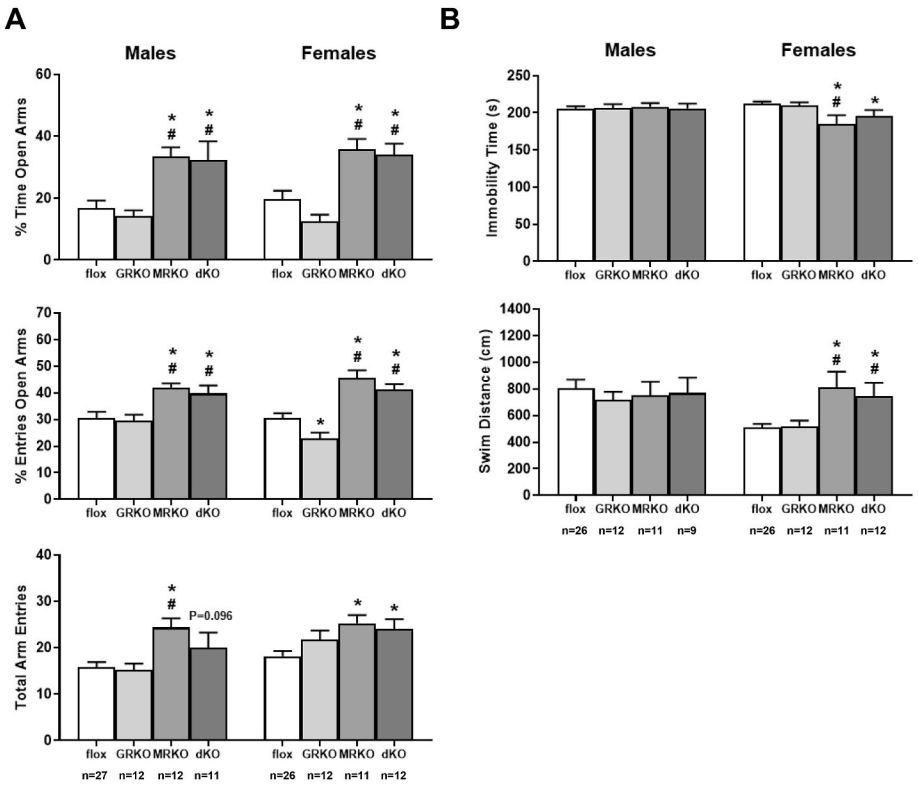

Fig. 4. Anxiety-like behavior and stress adaptation behavior in male and female GR$^{Emx1\text{-cre}}$, MR$^{Emx1\text{-cre}}$, and GRMR$^{Emx1\text{-cre}}$ mice. (**A**) The elevated plus maze test was performed on male and female flox control and knockout mice. Shown are percent time mice spent in the open arms (upper graph), percent entries of mice into the open arms (middle graph), and total number of arm entries (lower graph). Data are mean ± SEM (n values in figure). One-way ANOVAs followed by Fisher's PLSD tests were performed separately on males and females to determine significance [open arm time males: $F(3,58) = 8.162$, $p = 0.0001$; females: $F(3,57) = 10.58$, $p < 0.0001$; open arm entries males: $F(3,58) = 5.411$, $p = 0.0024$; females: $F(3,57) = 17.06$, $p < 0.0001$; total arm entries males: $F(3,58) = 5.231$, $p = 0.0029$; females: $F(3,57) = 4.322$,$p = 0.0082$]. (**B**) The forced swim test was performed on male and female flox control and knockout mice. Shown are time spent immobile (upper graph) and swimming distance (lower graph) for the mice. Data are mean ± SEM (n values in figure). One-way ANOVAs followed by Fisher's PLSD tests were performed separately on males and females to determine significance [immobility time females: $F(3,57) = 4.043$, $p = 0.0112$; swim distance females: $F(3,57) = 5.515$, $p = 0.0021$]. *P < 0.05 for GRKO, MRKO, and dKO compared to flox control within sex group. $^{\#}$P < 0.05 for MRKO and dKO compared to GRKO within sex group.

contrast, female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice demonstrated significantly less immobile floating and more swimming activity than flox control mice, suggesting an altered stress adaptation response. Since the female GRMR$^{Emx1-cre}$ mice show the same alteration in stress coping behavior as the female MR$^{Emx1-cre}$ mice which have elevated levels of GR, we conclude that the deficiency in MR signaling is responsible for the behavioral phenotype. Collectively, these data suggest that hippocampal MR acts in a sex-specific manner to promote a switch from active coping to passive coping in female mice in the face of an acute stressor.

Sociability was examined in male and female GR$^{Emx1-cre}$, MR$^{Emx1-cre}$, and GRMR$^{Emx1-cre}$ mice using the social approach test (Fig. 5A). Mice were placed in the center chamber of a 3-chambered box and given a choice between being in the proximity of an unfamiliar mouse (stranger) in one side chamber or a non-social novel object (an empty cage) in the other side chamber. Measures were taken of the time the mice spent in the side chamber containing the stranger mouse versus the empty cage as well as the time spent in proximity to the stranger mouse versus the empty cage. Overall repeated measures ANOVAs (with factors genotype, sex, and side) indicated a main effect of genotype [$F(3,114) = 7.08$, $p = 0.0002$] and a main effect of side [$F(1,114) = 56.63$, $p < 0.0001$] for time in each side. For time in proximity to each cage, which provides a more direct index of social investigation, not only were main effects of genotype [$F(3,114) = 3.95$, $p = 0.0101$] and side [$F(1,114) = 58.45$, $p < 0.0001$] detected but also significant interactions between genotype and side [$F(3,114) = 2.94$, $p = 0.0362$] and between sex and side [$F(1,114) = 4.34$, $p = 0.0396$], indicating that the degree of sociability was dependent upon both genotype and sex. For entries into each side, a main effect of side [$F(1,114) = 40.82$, $p < 0.0001$] was detected. Post-hoc testing indicated that both the male and female flox control mice showed the expected preference for being near the stranger mouse as indicated by the increased time spent in the stranger mouse side and in proximity to the stranger mouse compared to the empty cage. A similar preference for the stranger mouse was also observed in female mice lacking GR, MR, or both receptors in the hippocampus. In marked contrast, male GR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice showed no preference for the stranger mouse in either the side time or proximity time measurements, and male MR$^{Emx1-cre}$ mice showed no preference for the stranger mouse in the proximity time measurement. These alterations in social preference observed for the male knockout mice were not due to general changes in activity since no significant genotype effects were observed for the number of entries into the side chambers. Taken together, these data suggest that hippocampal GR and MR function in sex-specific manner to maintaining appropriate levels of sociability in male mice.

Fear-motivated learning and memory was investigated in male and female knockout mice using the conditioned fear test (Fig. 5B). Mice were trained on day 1 by placing them in the conditioning chamber, allowing them to explore for 2 min, and then exposing them to 3 shock-tone pairings. Contextual memory was evaluated on day 2 and day 16 by placing the mice back into the original conditioning chamber and measuring the levels of freezing, an index of learning, across a 5 min session. Male data for context learning (and cue learning below) has been previously published but is shown again for comparison to the female data (Oakley et al., 2021). Overall repeated measures ANOVAs

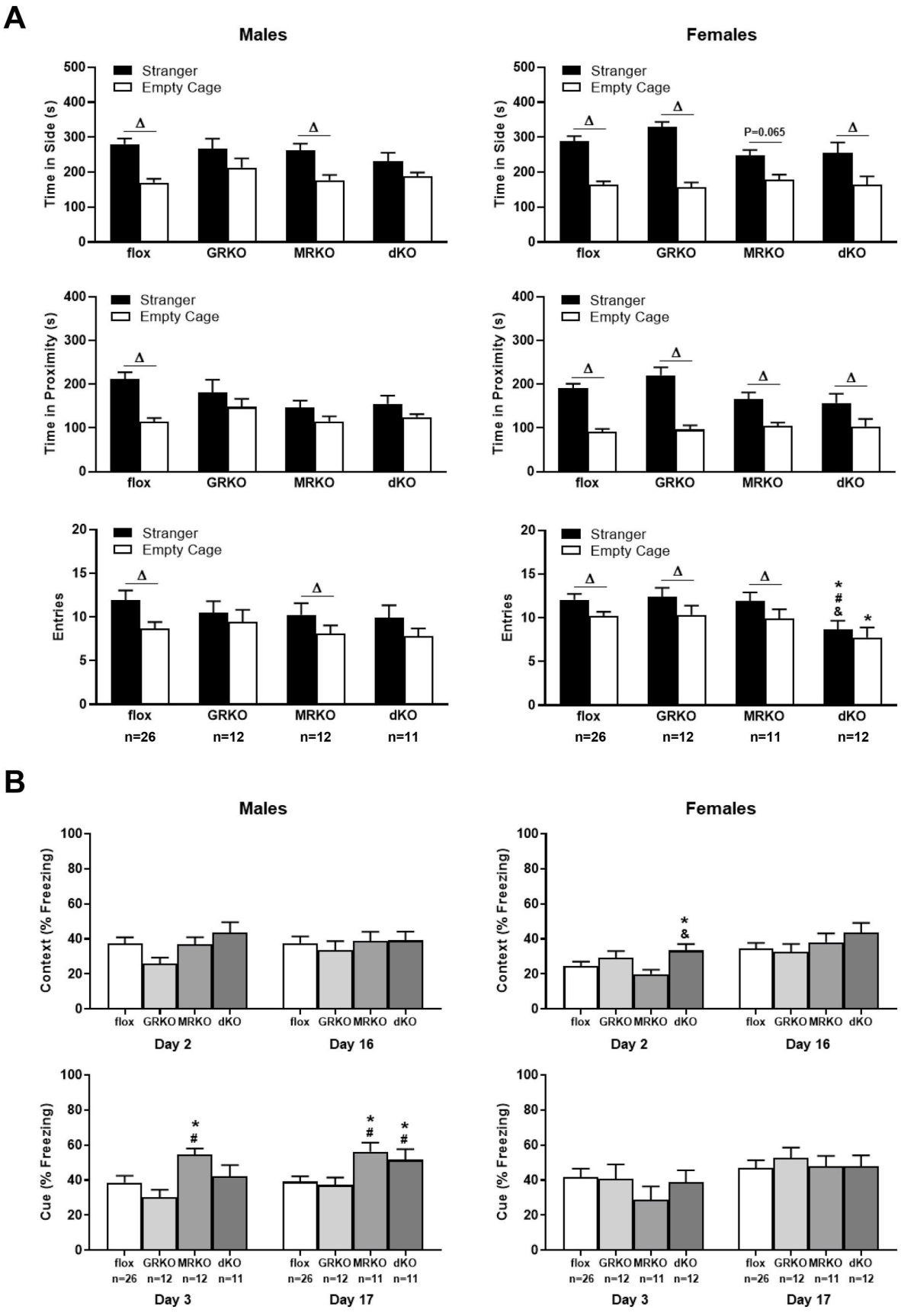

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Fig. 5. Social approach and fear-motivated learning behavior in male and female GR$^{Emx1\text{-cre}}$, MR$^{Emx1\text{-cre}}$, and GRMR$^{Emx1\text{-cre}}$ mice. (**A**) The social approach test was performed on male and female flox control and knockout mice. Shown are time spent in the stranger mouse side (upper graphs), time in proximity to the stranger mouse (middle graphs), and number of entries into the stranger mouse side (lower graphs) versus the empty cage. Data are mean ± SEM (n values in figure). Repeated measures ANOVAs followed by Fisher's PLSD test were performed separately on males and females to determine significance [time in side males: main effect of side, $F(1,57) = 15.53$, $p = 0.0002$; females: main effect of genotype, $F(3,57) = 4.69$, $p = 0.0054$, main effect of side, $F(1,57) = 47.45$, $p < 0.0001$; time in proximity males: main effect of genotype, $F(3,57) = 2.969$, $p = 0.0393$, main effect of side, $F(1,57) = 11.71$, $p = 0.0012$; females: main effect of side, $F(1,57) = 69.65$, $p < 0.0001$; and entries males: main effect of side, $F(1,57) = 19.27$, $p < 0.0001$; females: main effect of genotype, $F(3,57) = 3.067$, $p = 0.0351$, main effect of side, $F(1,57) = 23.31$, $p < 0.0001$]. (**B**) The conditioned fear test was performed on male and female flox control and knockout mice. Context-dependent learning was evaluated by measuring levels of freezing across a 5-min session on Day 2 and Day 16 (upper graphs). Cue-dependent learning was evaluated by measuring levels of freezing during a 3-min presentation of an acoustic stimulus on Day 3 and Day 17 (lower graphs). Data are mean ± SEM (n values in figure). One-way ANOVAs followed by Fisher's PLSD tests were performed separately on males and females to determine significance [context females Day 2: $F(3,57) = 2.836$, $p = 0.0461$; cue males Day 3: $F(3,57) = 3.924$, $p = 0.0129$; cue males Day 17: $F(3,56) = 4.145$, $p = 0.0101$]. $\Delta < 0.05$ for within genotype comparison to empty cage side. $^{*}P < 0.05$ for MRKO and dKO compared to flox control, $^{\#}P < 0.05$ for MRKO and dKO compared to GRKO, and $^{\&}P < 0.05$ for dKO compared to MRKO within sex group.

(with factors genotype, sex, and test day) revealed a main effect of test day [$F(1,113) = 15.48$, $p = 0.0001$] and a significant interaction between sex and test day [$F(1,113) = 9.84$, $p = 0.0022$] on contextual learning. Post-hoc testing indicated that knockout of GR, MR, or both receptors in the male hippocampus had no significant effects on fear-motivated contextual learning. The female GR$^{Emx1-\text{cre}}$ and MR$^{Emx1-\text{cre}}$ mice also exhibited normal levels of contextual learning. Interestingly, the female GRMR$^{Emx1-\text{cre}}$ mice displayed increased contextual learning during the day 2 test, but this effect was transient and not observed during the day 16 test for memory retention.

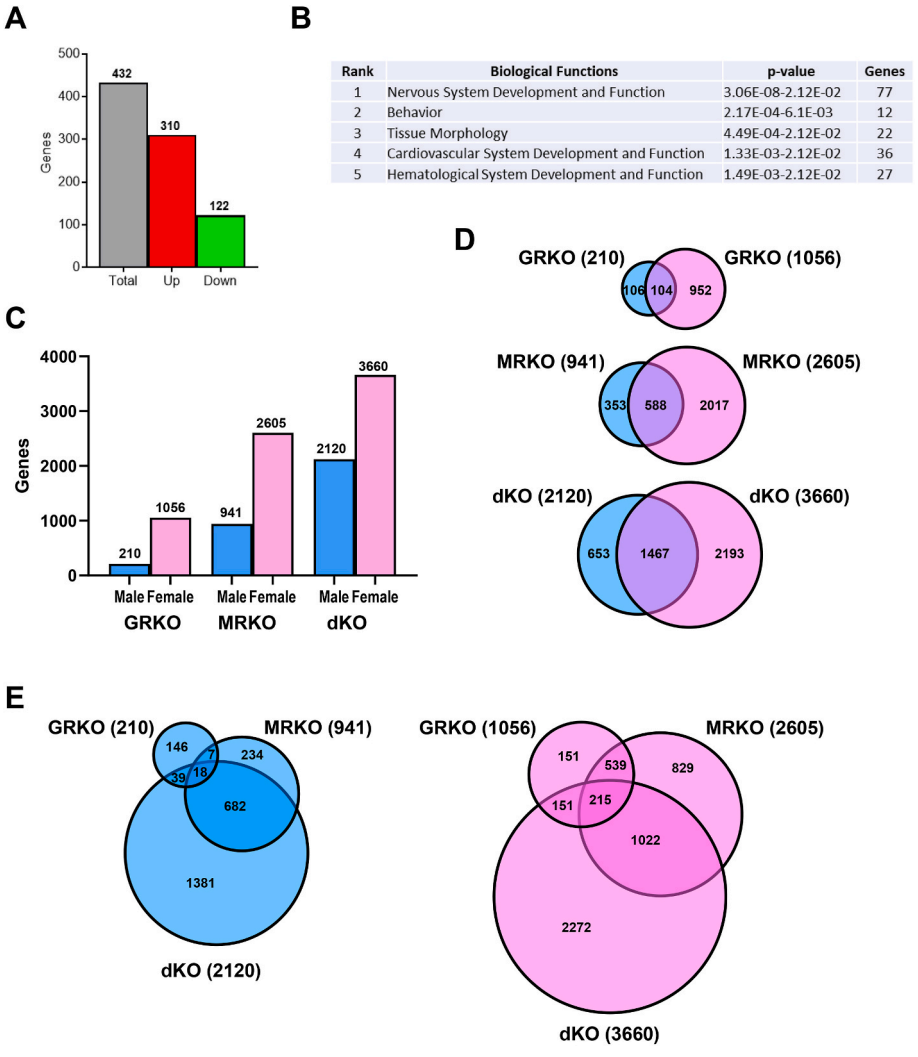


Fig. 6. Global gene expression profiles in male and female GR$^{Emx1\text{-cre}}$, MR$^{Emx1\text{-cre}}$, and GRMR$^{Emx1\text{-cre}}$ mice. A genome-wide microarray was performed on hippocampal RNA from ~2.5-month old male and female flox control and knockout mice. (**A**) Total number of differentially expressed genes in the hippocampus of female flox control mice compared to male flox control mice. (**B**) Top IPA biological functions most significantly associated with the 432 differentially expressed genes in the male and female flox control hippocampus. The range in p-values reflects the p-values of the multiple lower-level functions that comprise the indicated high-level functional category. (**C**) Total number of dysregulated genes in the hippocampus of male (blue) and female (pink) knockout mice compared to flox control mice of same sex. (**D**) Within genotype comparisons of male and female GR$^{Emx1-\text{cre}}$, MR$^{Emx1-\text{cre}}$, and GRMR$^{Emx1-\text{cre}}$ dysregulated genes. (**E**) Within sex comparisons of male and female GR$^{Emx1-\text{cre}}$, MR$^{Emx1-\text{cre}}$, and GRMR$^{Emx1-\text{cre}}$ dysregulated genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fear-motivated cue learning was evaluated on day 3 and day 17 by placing the mice into a modified conditioning chamber and measuring the levels of freezing during the 3-min acoustic stimulus. Overall repeated measures ANOVAs (with factors genotype, sex, and test day) revealed a main effect of test day [$F(1,113) = 13.77$, $p = 0.0003$] and a significant interaction between genotype and sex [$F(3,113) = 3.31$, $p = 0.0227$] on cue learning. Post-hoc testing indicated that male $MR^{Emx1-cre}$ mice exhibited higher levels of freezing during both the day 3 and day 17 tests and male $GRMR^{Emx1-cre}$ mice exhibited increased freezing during the day 17 test. Since the male $GRMR^{Emx1-cre}$ mice show a cue learning phenotype similar to the male $MR^{Emx1-cre}$ mice which have elevated levels of GR, we conclude that the loss of MR signaling is responsible for the enhanced cue learning behavior in the male mice. In contrast to our findings in male mice, no significant differences in fear-motivated cue learning were observed for the female single and double knockout mice. Taken together, these findings suggest that MR signaling acts in a sex-specific manner to regulate fear-motivated cue learning in male mice.

3.5. *Hippocampal GR and MR signaling regulate the hippocampal transcriptome in both a sex-dependent and independent manner*

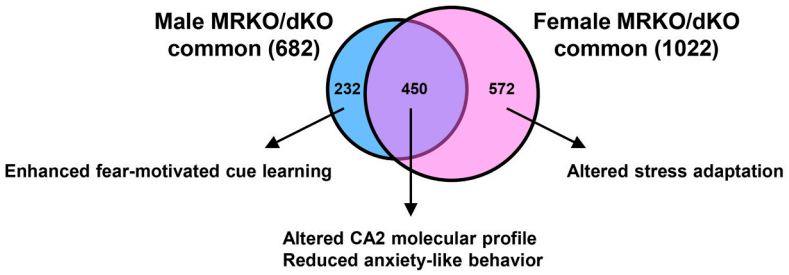
To determine how a deficiency in GR, MR, or both receptors altered the hippocampal transcriptome, we performed a genome-wide microarray on RNA isolated from the hippocampus of male and female flox control, $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ mice. The male data has been previously published as a separate analysis (Oakley et al., 2021). For the current study, the male and female data were combined and analyzed together. A comparison of the male and female flox control hippocampi revealed 432 differentially expressed genes, with 310 exhibiting increased and 122 decreased expression in the female mice (Fig. 6A). Ingenuity pathway analysis (IPA) was performed to investigate the biological functions enriched with these 432 sex biased genes. The top two functions associated with these genes were Nervous System Development and Function and Behavior (Fig. 6B), consistent with well-established sex differences in brain development and function (Bundy et al., 2017; Yagi and Galea, 2019). Knockout of hippocampal GR, MR, or both receptors in male mice resulted in the dysregulation of 210, 941, and 2120 genes, respectively (Fig. 6C). In contrast, knockout of hippocampal GR, MR, or both receptors in female mice resulted in the dysregulation of a much greater number of 1056, 2605, and 3660 genes, respectively. Despite this pronounced sex difference in the total number of dysregulated genes, the overall pattern of gene changes in the male and female knockout mice was similar. In both sexes, loss of GR resulted in the fewest number of gene changes, loss of MR resulted in a moderate number of gene changes, and the concurrent loss of both receptors resulted in the greatest number of dysregulated genes.

Within genotype comparisons were performed on the dysregulated genes to identify the sex-dependent and independent gene changes (Fig. 6D). For each knockout mouse model there was substantial overlap of the male dysregulated genes with the female dysregulated genes, suggesting that a deficiency in GR and/or MR has major sex independent effects in the hippocampus. However, this comparison also revealed the presence of many sex-specific gene changes. The male and female $GR^{Emx1-cre}$ mice exhibited 106 and 952 unique gene changes, respectively; the male and female $MR^{Emx1-cre}$ mice exhibited 353 and 2017 unique gene changes, respectively; and the male and female $GRMR^{Emx1-cre}$ mice exhibited 653 and 2193 unique gene changes, respectively. The large number of gene changes occurring only in males or only in females suggests that a deficiency in GR and/or MR also has prominent sex-dependent effects in the hippocampus, with the female hippocampus being especially sensitive to imbalances in these two receptors.

We next performed within sex comparisons of the $GR^{Emx1-cre}$, $MR^{Emx1-cre}$, and $GRMR^{Emx1-cre}$ dysregulated genes (Fig. 6E). This analysis identified 2 major patterns of gene dysregulation conserved in both sexes that are directly relevant to the main phenotypes observed in the male and female single and double knockout mice. The first major pattern was that the largest overlap among the 3 sets of dysregulated genes occurred between the $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice. There were 682 $MR^{Emx1-cre}$/$GRMR^{Emx1-cre}$ common gene changes in males and 1022 $MR^{Emx1-cre}$/$GRMR^{Emx1-cre}$ common gene changes in females (Fig. 6E). This pronounced overlap is consistent with the large number of phenotypes shared between the $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mouse models, including the altered molecular profile of CA2 neurons (Fig. 2), reduced anxiety-like behavior (Fig. 4A), altered stress adaptation (Fig. 4B), and enhanced fear-motivated cue learning (Fig. 5B). To elucidate the specific gene sets responsible for these shared phenotypes, we compared the 682 male $MR^{Emx1-cre}$/$GRMR^{Emx1-cre}$ common genes with the 1022 female $MR^{Emx1-cre}$/$GRMR^{Emx1-cre}$ common genes (Fig. 7A). This analysis identified 450 genes that were commonly altered in the hippocampus of both male and female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice. IPA was performed on these genes and revealed strong associations with axonal guidance, opioid, ephrin receptor, and CREB signaling pathways and with the pathology Neurological Disease (Fig. 7B). The dysregulation of these genes and their respective signaling pathways likely underlies the altered molecular profile of CA2 neurons and the reduced anxiety-like behavior that were observed in both the male and female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice. The comparison also revealed 232 sex biased genes that were uniquely dysregulated only in the male $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ hippocampi (Fig. 7A). IPA of this gene set revealed strong associations with cAMP, semaphorin, endocannabinoid, and axonal guidance signaling pathways (Fig. 7B). Neurological Disease was the top-ranked pathology enriched with this gene set (Fig. 7B). The dysregulation of these genes and their respective signaling pathways likely underlies the enhanced fear-motivated cue learning observed only in the male $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice. Finally, this comparison identified 572 sex biased genes that were uniquely dysregulated only in the female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ hippocampi (Fig. 7A). The IPA pathways most significantly enriched with these genes included synaptogenesis, ephrin receptor, and SNARE signaling pathways (Fig. 7B). Neurological Disease and Psychological Disorders were among the top disease annotations associated with this gene set (Fig. 7B). The dysregulation of these genes and their respective signaling pathways likely underlies the altered stress adaptation behavior observed only in the female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice.

The occurrence of gene changes in both the $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mouse models suggests that these genes depend primarily on MR signaling for their appropriate expression levels in the hippocampus. To distinguish genes that are direct targets of MR from genes that are dysregulated in a secondary manner, we performed IPA Upstream Regulator analysis which predicts the molecules responsible for the gene expression changes. This analysis identified 82 potential MR target genes from the 450 male and female common gene set (Fig. 7C and Table S1). Interestingly, no potential MR target genes were identified from the 232 male specific gene set and only 32 were retrieved from the 572 female specific gene set, suggesting MR targeting of these sex-specific genes involves novel modes of regulation. We further analyzed the large set of 82 potential MR target genes common to the male and female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice and found that Behavior was one of the top ranked IPA functions enriched with this gene set (Fig. 7C). Provocatively, within the broad Behavior category, Anxiety-like Behavior and Anxiety were two of the highest ranked specific behavioral annotations associated with the 82 MR target genes (Fig. 7C). This finding is consistent with the altered anxiety-like behavior observed in both the male and female $MR^{Emx1-cre}$ and $GRMR^{Emx1-cre}$ mice (Fig. 4A). Of the 82 MR target genes, 18 were associated with Behavior and are shown overlaid with the male $MR^{Emx1-cre}$, male $GRMR^{Emx1-cre}$, female $MR^{Emx1-cre}$, and female $GRMR^{Emx1-cre}$ expression data (Fig. 7C and D). All but one of the genes (Mup1) were regulated in the same direction in each of the knockout

A



B

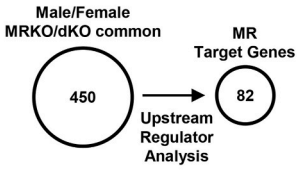
Nervous System Signaling Pathways

Male MRKO/dKO Unique (232)	p-value	Genes
1. Dopamine-DARPP32 Feedback in cAMP Signaling	6.46E-03	7
2. Semaphorin Neuronal Repulsive Signaling Pathway	8.13E-03	6
3. Endocannabinoid Neuronal Synapse Pathway	9.12E-03	6
4. GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	1.29E-02	5
5. Axonal Guidance Signaling	1.62E-02	12
Male and Female MRKO/dKO Common (450)		
1. Axonal Guidance Signaling	1.58E-04	26
2. Opioid Signaling Pathway	3.39E-04	17
3. GNRH Signaling	2.04E-03	12
4. Ephrin Receptor Signaling	3.55E-03	12
5. CREB Signaling in Neurons	3.72E-03	25
Female MRKO/dKO Unique (572)		
1. Synaptogenesis Signaling Pathway	1.74E-03	19
2. Ephrin Receptor Signaling	1.51E-02	12
3. SNARE Signaling Pathway	3.89E-02	8
4. Agrin Interactions at Neuromuscular Junction	4.79E-02	5

Diseases and Disorders

Male MRKO/dKO Unique (232)	p-value	Genes
1. Neurological Disease	2.14E-05-1.22E-02	162
2. Organismal Injury and Abnormalities	2.14E-05-1.22E-02	209
3. Gastrointestinal Disease	7.2E-05-1.22E-02	185
4. Cancer	8.84E-05-1.22E-02	208
5. Hepatic System Disease	1.16E-04-1.11E-03	123
Male and Female MRKO/dKO Common (450)		
1. Cancer	1.8E-09-1.1E-02	408
2. Gastrointestinal Disease	1.8E-09-9.72E-03	376
3. Organismal Injury and Abnormalities	1.8E-09-1.11E-02	413
4. Neurological Disease	2.04E-07-1.1E-02	317
5. Dermatological Diseases and Conditions	2.33E-06-1.06E-02	320
Female MRKO/dKO Unique (572)		
1. Neurological Disease	4.02E-05-2.95E-02	163
2. Organismal Injury and Abnormalities	4.02E-05-2.95E-02	434
3. Psychological Disorders	4.02E-05-2.95E-02	89
4. Respiratory Disease	4.24E-05-2.95E-02	133
5. Cardiovascular Disease	3.8E-04-2.95E-02	59

C



Rank	Biological Functions	p-value	Genes
1	Hematological System Development and Function	2.5E-06-9.29E-03	33
2	Lymphoid Tissue Structure and Development	2.5E-06-6.08E-03	21
3	Behavior	3.5E-06-8.56E-03	18
4	Organismal Development	3.87E-06-9.29E-03	50
5	Nervous System Development and Function	1.48E-05-9.29E-03	27

Rank	Behavior Annotation	p-value	Genes
1	Learning	3.50E-06	14
2	Anxiety-like behavior	8.48E-05	5
3	Memory	1.90E-04	9
4	Spontaneous pain behavior	2.12E-04	2
5	Memory consolidation	3.26E-04	3
6	Anxiety	6.25E-04	7

D

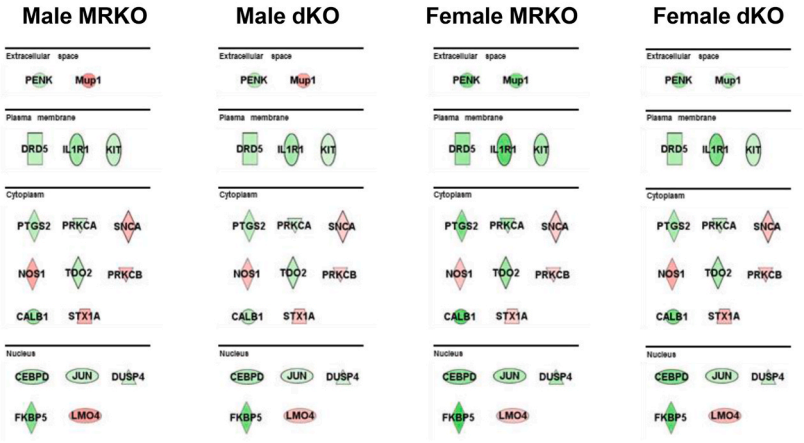


Fig. 7. Gene changes and signaling pathways underlying the sex-dependent and independent phenotypes common to the MR$^{Emx1\text{-}cre}$ and GRMR$^{Emx1\text{-}cre}$ mice. (**A**) Comparison of the 682 male MR$^{Emx1-cre}$/GRMR$^{Emx1-cre}$ common genes with the 1022 female MR$^{Emx1-cre}$/GRMR$^{Emx1-cre}$ common genes. Phenotypes observed in this study that are unique to the male, common to the male and female, or unique to the female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice are depicted under the 232 male unique, 450 male and female common, and 572 female unique gene sets, respectively. (**B**) Top IPA nervous system signaling pathways (left panel) and diseases and disorders (right panel) most significantly associated with the 232 male MR$^{Emx1-cre}$/GRMR$^{Emx1-cre}$ unique, 450 male and female MR$^{Emx1-cre}$/GRMR$^{Emx1-cre}$ common, and 572 female MR$^{Emx1-cre}$/GRMR$^{Emx1-cre}$ unique genes dysregulated in the hippocampus. (**C**) IPA Upstream Regulator analysis of the 450 male and female MR$^{Emx1-cre}$/GRMR$^{Emx1-cre}$ common genes identified 82 potential MR target genes (left panel). Top IPA biological functions and behavioral annotations associated with the 82 MR target genes (right panels). (**D**) The 18 MR target genes associated with Behavior are shown overlaid with the male MR$^{Emx1-cre}$, male GRMR$^{Emx1-cre}$, female MR$^{Emx1-cre}$, and female GRMR$^{Emx1-cre}$ expression data. Red and green colors correspond to up-regulation and down-regulation, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mouse models. Strikingly, 7 of the 18 Behavior genes have been associated with anxiety: Penk, Il1r1, Snca, Nos1, Tdo2, Stx1a, and Fkbp5. MR deficiency and the consequent alteration in expression of these 7 MR target genes may serve as the proximal event leading to the anxiolytic phenotype observed in the MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice. RTPCR validation of these MR target genes commonly altered in both the male and female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ hippocampus was performed using an independent set of mice on Nos1, Stx1a, and Fkbp5 (Fig. 8). Significant upregulation of Nos1 and Stx1a mRNA and downregulation of Fkbp5 mRNA were observed in both the male and female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ hippocampus, consistent with the microarray results.

The second major pattern of gene dysregulation uncovered by the within sex comparisons was that the largest number of unique gene changes occurred in the male and female GRMR$^{Emx1-cre}$ hippocampi (Fig. 6E). There were 1381 gene changes unique to the male GRMR$^{Emx1-cre}$ hippocampus and 2272 gene changes unique to the female GRMR$^{Emx1-cre}$ hippocampus. These dysregulated genes and their respective signaling pathways are likely responsible for the extensive neurodegeneration that was only observed in the dentate gyrus of the double knockout mice (Fig. 3). To elucidate the gene changes underlying the neurodegeneration, we compared the 1381 male GRMR$^{Emx1-cre}$ unique genes with the 2272 female GRMR$^{Emx1-cre}$ unique genes (Fig. 9A). We focused our attention on the 495 genes that were commonly altered in the male and female GRMR$^{Emx1-cre}$ hippocampus since the neurodegeneration occurred in both sexes. IPA demonstrated that the 495 genes were strongly associated with axonal guidance, endocannabinoid, opioid, and CREB signaling pathways (Fig. 9B). In addition, they were strongly associated with Neurological Disease and Inflammatory Disease (Fig. 9B). The occurrence of these gene changes only in the double knockout hippocampus suggests their appropriate expression depends on the actions of both GR and MR. IPA Upstream Regulator analysis of the 495 genes identified 99 as potential direct targets of GR and MR (Fig. 9C and Table S2). Cell Death and Survival was the top ranked IPA function associated with these 99 target genes and Cellular Growth and Proliferation was the 5th ranked annotation (Fig. 9C). This finding is consistent with our previous demonstration that both an increase in granule neuron death and a decrease in neurogenesis contribute to the neuronal loss in the GRMR$^{Emx1-cre}$ dentate gyrus (Oakley et al., 2021). Of the 99 GR/MR target genes, 63 were associated with Cell Death and Survival and 70 with Cellular Growth and Proliferation (Fig. 9C). Together, they comprise 75 distinct GR/MR target genes that are shown overlaid with the male and female GRMR$^{Emx1-cre}$ expression data in Fig. 9D. The majority of these gene changes (63 of 75) occur in the same direction in both the male and female hippocampus. The altered expression of one or more of these genes, triggered by the concurrent loss of GR and MR, may serve as the proximal event leading to the profound neurodegeneration that develops in the male and female GRMR$^{Emx1-cre}$ dentate gyrus. RTPCR validation of these GR/MR target

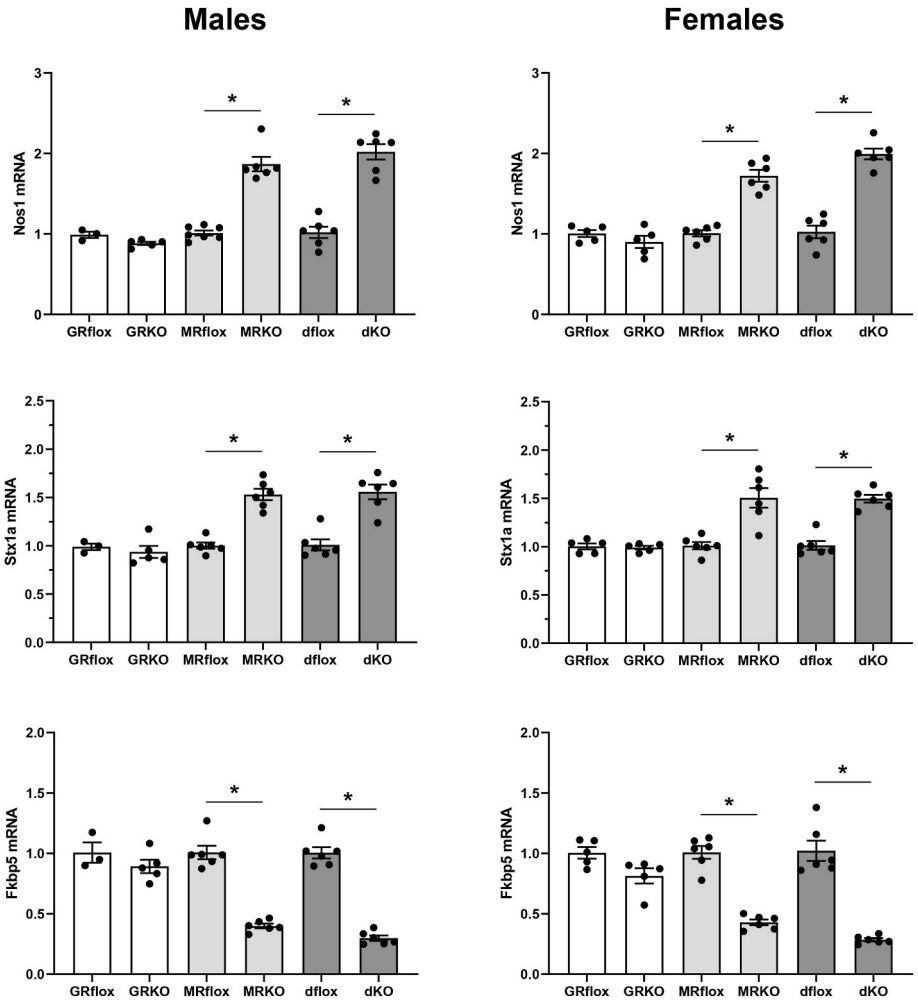
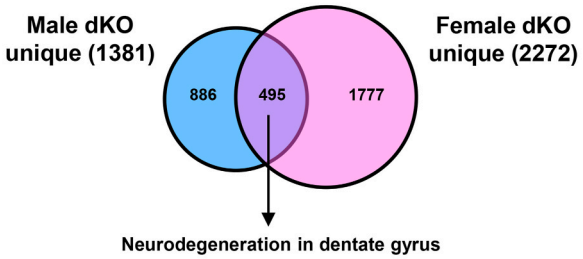


Fig. 8. Validation of hippocampal gene changes common to the male and female MR$^{Emx1\text{-}cre}$ and GRMR$^{Emx1\text{-}cre}$ mice. RNA was isolated from the hippocampus of an independent set of approximately 2- to 4-month old male and female flox control and knockout mice. RTPCR of nitric oxide synthase 1 (*Nos1*), syntaxin 1A (*Stx1a*), and FK506 binding protein 51 (*Fkbp5*) mRNA. Data are mean ± SEM (n = 3–7 mice per group). One-way ANOVAs followed by Tukey post-hoc tests were performed separately on males and females to determine significance. *P < 0.05 for MRKO compared to MRflox and dKO compared to dflox.

A



B

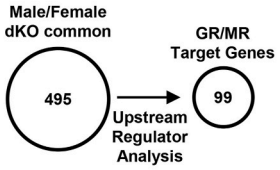
Nervous System Signaling Pathways

Male dKO Unique (886)	p-value	Genes
1. Ephrin Receptor Signaling	5.25E-03	18
2. Neurovascular Coupling Signaling Pathway	2.14E-02	17
3. Regulation of Actin-based Motility by Rho	2.63E-02	10
4. Agrin Interactions at Neuromuscular Junction	3.47E-02	7
5. Synaptogenesis Signaling Pathway	4.37E-02	21
Male and Female dKO Common (495)		
1. Axonal Guidance Signaling	9.55E-04	25
2. Endocannabinoid Neuronal Synapse Pathway	1.51E-03	11
3. Endocannabinoid Developing Neuron Pathway	1.66E-03	10
4. Opioid Signaling Pathway	1.82E-03	16
5. CREB Signaling in Neurons	2.19E-03	27
Female dKO Unique (1777)		
1. Regulation of Actin-based Motility by Rho	1.32E-03	20
2. Neuroinflammation Signaling Pathway	1.38E-03	41
3. Ephrin B Signaling	1.45E-03	15
4. Oxytocin In Brain Signaling Pathway	3.98E-03	28
5. Axonal Guidance Signaling	5.37E-03	61

Diseases and Disorders

Male dKO Unique (886)	p-value	Genes
1. Cancer	2.39E-04-1.21E-02	713
2. Gastrointestinal Disease	2.39E-04-1.21E-02	661
3. Organismal Injury and Abnormalities	2.39E-04-1.21E-02	728
4. Inflammatory Response	2.75E-04-9.29E-03	111
5. Skeletal and Muscular Disorders	2.75E-04-1.21E-02	174
Male and Female dKO Common (495)		
1. Inflammatory Response	8.31E-08-2.91E-03	146
2. Neurological Disease	8.31E-08-2.76E-03	186
3. Organismal Injury and Abnormalities	8.31E-08-2.99E-03	423
4. Inflammatory Disease	7.13E-07-2.1E-03	113
5. Cancer	1.72E-06-2.85E-03	416
Female dKO Unique (1777)		
1. Inflammatory Response	1.74E-05-7E-03	322
2. Cancer	2.71E-05-7.1E-03	1408
3. Gastrointestinal Disease	2.71E-05-6.89E-03	555
4. Organismal Injury and Abnormalities	2.71E-05-7.1E-03	1432
5. Renal and Urological Disease	2.9E-05-6.41E-03	22

C



Rank	Biological Functions	p-value	Genes
1	Cell Death and Survival	1.98E-15-4.47E-05	63
2	Cellular Movement	8.54E-15-4.4E-05	57
3	Cell-To-Cell Signaling and Interaction	5.4E-13-3.25E-05	59
4	Cellular Development	6.01E-12-4.5E-05	73
5	Cellular Growth and Proliferation	6.01E-12-4.01E-05	70

D

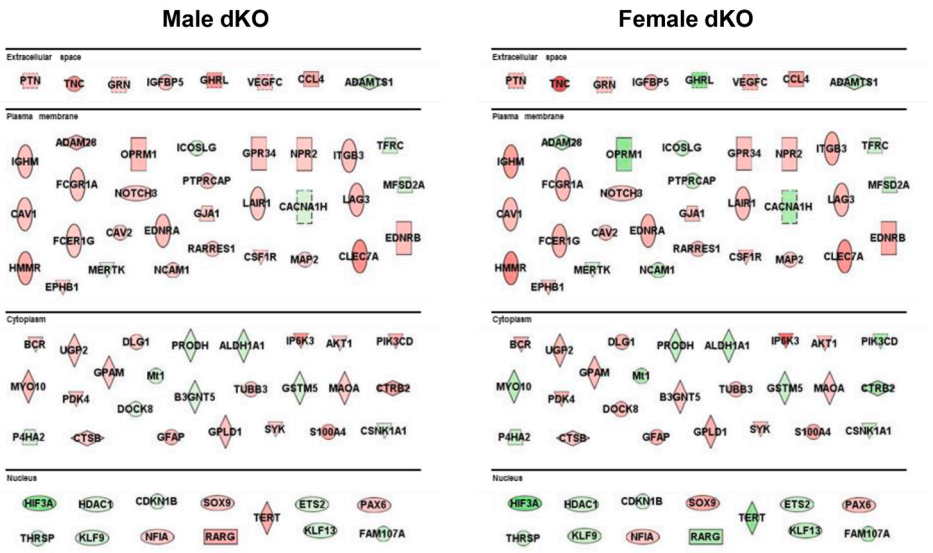


Fig. 9. Gene changes and signaling pathways underlying the sex independent neurodegeneration phenotype unique to the GRMR$^{Emx1\text{-cre}}$ mice. (**A**) Comparison of the 1381 male GRMR$^{Emx1-cre}$ unique genes with the 2272 female GRMR$^{Emx1-cre}$ unique genes. The neurodegeneration phenotype observed in this study that is common to the male and female GRMR$^{Emx1-cre}$ mice is depicted under the 495 male and female common gene set. (**B**) Top IPA nervous system signaling pathways (left panel) and diseases and disorders (right panel) most significantly associated with the 886 male GRMR$^{Emx1-cre}$ unique, 495 male and female GRMR$^{Emx1-cre}$ common, and 1777 female GRMR$^{Emx1-cre}$ unique genes dysregulated in the hippocampus. (**C**) IPA Upstream Regulator analysis of the 495 male and female GRMR$^{Emx1-cre}$ common genes identified 99 potential GR/MR target genes (left panel). Top IPA functions associated with the 99 GR/MR target genes (right panel). (**D**) The 75 GR and MR target genes that are associated with Cell Death and Survival and/or Cellular Growth and Proliferation are shown overlaid with the male and female GRMR$^{Emx1-cre}$ expression data. Red and green colors correspond to up-regulation and down-regulation, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes commonly altered in both the male and female GRMR$^{Emx1-cre}$ hippocampus was performed using an independent set of mice on Ednra, PTN, and Klf9 (Fig. 10). The male data for Ednra and PTN has been previously published but are shown again for comparison with the female data (Oakley et al., 2021). Significant upregulation of Ednra and PTN mRNA and downregulation of Klf9 mRNA were detected in both the male and female GRMR$^{Emx1-cre}$ hippocampus, consistent with the microarray results. A small upregulation in PTN mRNA was also measured in the female GR$^{Emx1-cre}$ hippocampus but a significantly greater increase occurred in the double knockout. Taken together, our global gene expression data has identified male and female genetic signatures and candidate genes that underlie the sex-dependent and independent phenotypes that result from alterations in GR and MR signaling in the hippocampus.

4. Discussion

We investigated the sex-dependent and independent effects of imbalanced GR/MR signaling in the hippocampus using male and female mice with conditional knockout of hippocampal GR, MR, or both receptors. We report that a deficiency in hippocampal MR signaling altered the molecular identity of CA2 neurons and anxiety-like behavior in both sexes but affected fear-motivated cue learning only in males and stress adaptation behavior only in females. Reductions in hippocampal GR and/or MR signaling impacted sociability only in male mice. The concurrent deficiency in hippocampal GR and MR signaling resulted in extensive neurodegeneration in the dentate gyrus in both male and female mice. Global gene expression analysis of the knockout hippocampi revealed a marked expansion in the number of dysregulated genes in female mice but remarkable conservation of the overall patterns of gene changes in both sexes. Within and across sex comparisons of the dysregulated genes resulted in the identification of GR and MR target genes whose altered expression may serve as proximal mediators of the observed phenotypes of the single and double knockout mice. These data define for the first time the sex-dependent and independent genetic, molecular, and functional signatures of GR/MR imbalances in the hippocampus.

A distinguishing feature of our study is the development and use of the GRMR$^{Emx1-cre}$ mouse model. The double knockout mice are essential for accurately assigning observed phenotypes in the single knockout mice to alterations in GR or MR signaling since the loss of one receptor can lead to the aberrant activity of the remaining receptor. Indeed, several laboratories have reported that a deficiency in hippocampal MR signaling results in the upregulation of GR in the hippocampus but the functional relevance of this change has not been described (McCann et al., 2021; Berger et al., 2006; Herman and Spencer, 1998; ter Horst

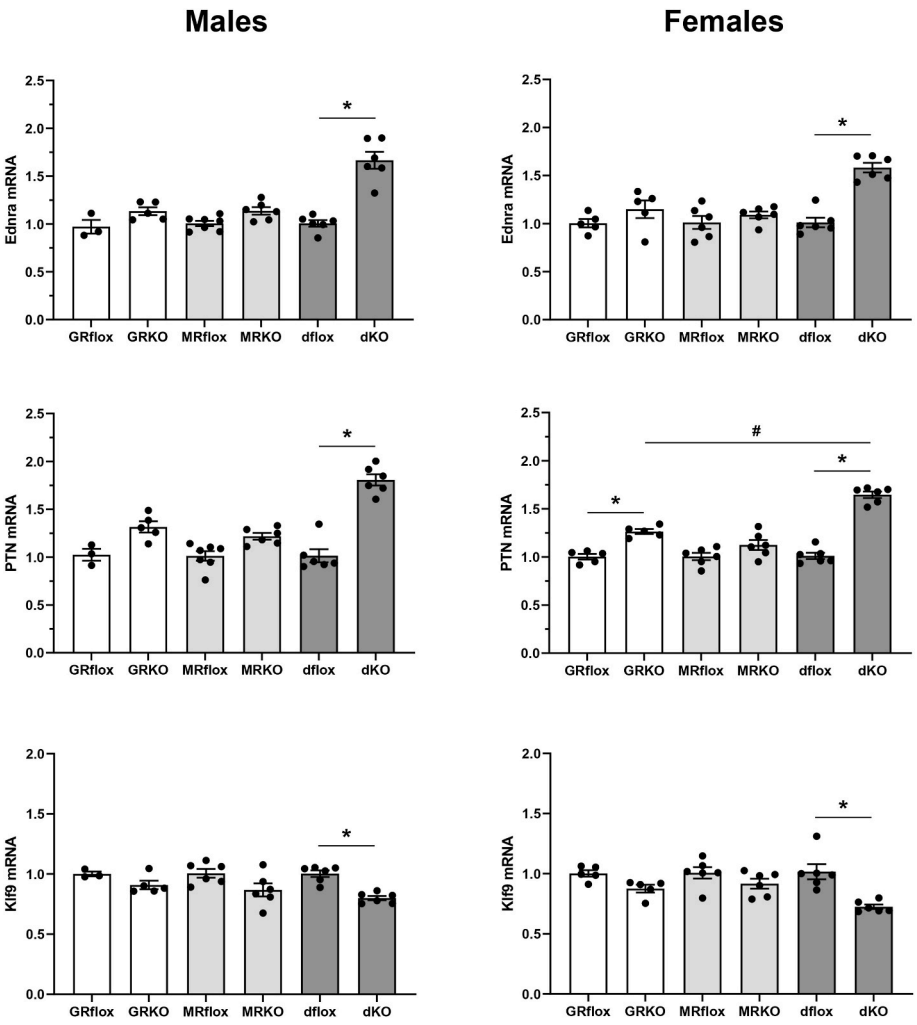


Fig. 10. Validation of hippocampal gene changes common to the male and female GRMR$^{Emx1\text{-}cre}$ mice. RNA was isolated from the hippocampus of an independent set of approximately 2- to 4-month old male and female flox control and knockout mice. RTPCR of endothelin receptor type A (*Ednra*), pleiotrophin (*PTN*), and Kruppel like factor 9 (*Klf9*) mRNA. Data are mean ± SEM (n = 3–7 mice per group). One-way ANOVAs followed by Tukey post-hoc tests were performed separately on males and females to determine significance. *P < 0.05 for GRKO compared to GRflox and dKO compared to dflox. #P < 0.05 for dKO compared to GRKO.

et al., 2012). Therefore, phenotypes observed in MR deficient mice may stem from the loss of MR signaling or the rise in aberrant GR signaling. The GRMR$^{Emx1-cre}$ mice distinguish between these two possibilities and reveal the molecular culprit behind the phenotype. By incorporating the double knockout mouse model in our experiments, we can conclude that the alterations in CA2 molecular identity, anxiety-like behavior, stress adaptation, and fear-motivated cue learning in the MR$^{Emx1-cre}$ mice are each due to the loss of MR signaling rather than aberrant GR signaling since the GRMR$^{Emx1-cre}$ mice mimicked the MR$^{Emx1-cre}$ mice for each of these phenotypes. Interestingly, the MR$^{Emx1-cre}$ mice exhibited not only greater overlap with the GRMR$^{Emx1-cre}$ mice than the GR$^{Emx1-cre}$ mice but also greater numbers of dysregulated genes and more abnormal phenotypes. These differences likely reflect our study being performed predominantly in mice at baseline when corticosterone levels are low and MR occupancy is favored.

The hippocampus, along with pituitary corticotropes and other brain regions such as the paraventricular nucleus of the hypothalamus, participates in the glucocorticoid negative feedback response of the HPA axis (Herman et al., 2020). Therefore, a surprising finding from our studies is that loss of hippocampal GR, MR, or both receptors did not lead to detectable changes in circadian corticosterone levels nor in peak acute restraint stress-induced corticosterone levels in either male or female mice (Fig. 1E) (Oakley et al., 2021). Since hippocampal control of HPA axis responses can be stimulus dependent (Herman et al., 2020), it will be important to examine HPA axis regulation in our single and double knockout mice in different stress paradigms. It will also be important to investigate whether the GR$^{Emx1-cre}$, MR$^{Emx1-cre}$, and GRMR$^{Emx1-cre}$ mice exhibit alterations in the kinetics of HPA axis activation and/or inactivation by negative feedback. While several studies have reported that loss of GR in the mouse forebrain can lead to dysregulation of the HPA axis (Boyle et al., 2005, 2006; Furay et al., 2008; Solomon et al., 2012), others have observed no alterations in HPA axis activity following depletion of GR in the hippocampus which is in agreement with our current findings (Vincent et al., 2013). Differences in testing regimens, the pattern of receptor deletion in the brain, and/or genetic background of the mice may account for these conflicting results. Compensatory mechanisms may also be at work in our single and double knockout mice that minimize disturbances in HPA axis regulation. Estrogens, for example, have been reported to normalize the HPA axis response to stress in aging male rodents by increasing GR expression in specific regions of the brain involved in glucocorticoid negative feedback (De Nicola et al., 2006; Ferrini et al., 1999).

Alterations in the GR/MR balance in the hippocampus of our knockout mice had several prominent sex-specific effects on hippocampal function. Depletion of GR, MR, or both receptors led to a reduction in social interaction in male mice but not females. These results are consistent with prior work showing that MR deficiency in the forebrain impaired social discrimination only in male mice (Ter Horst et al., 2014). Our data extend these findings by showing that glucocorticoid signaling through both GR and MR is critical for maintaining appropriate social motivation in male mice. This may have important implications for understanding stress-related mental illnesses with known social defects, such as schizophrenia, that occur more frequently in men than women (Thomas et al., 2010). Imbalanced GR/MR signaling in the hippocampus also had sex-specific effects on fear memory. Male MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice showed enhanced cue learning in the fear conditioning test whereas their female counterparts exhibited normal levels of learning. The male specific increase in fear-motivated cue learning has been reported in other mouse models lacking MR in the forebrain but the molecular culprit behind the phenotype remained ambiguous due to the observed upregulation of GR (Ter Horst et al., 2012). Our studies identify MR deficiency as the molecular trigger behind the fear memory enhancement since a similar phenotype was observed in both the MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ male mice. Shifting the GR/MR balance to favor more MR signaling may represent an improved approach for treating men with psychiatric disorders associated with exaggerated fear expressions.

Changes in the hippocampal GR/MR signaling balance also had sex-specific effects on stress adaptation in the forced swim test. Female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice exhibited a decrease in passive coping and an increase in active coping whereas their male counterparts did not. The common MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ phenotype indicates that the deficiency in MR, rather than aberrant GR signaling, is responsible for the observed alterations in stress coping behavior in the female mice. Previous studies utilizing selective GR and MR agonists and antagonists have revealed an important role for hippocampal GR in mediating the consolidation and retention of the passive coping response to the inescapable forced swim stressor (Molendijk and de Kloet, 2022). Interestingly, however, mice with genetic knockout of GR in the forebrain showed either no effect or an increase in the passive coping behavior in the forced swim test (Boyle et al., 2005; Solomon et al., 2012). In addition, a few recent reports using pharmacological agents to block receptor activity suggest that central MR may be involved in the passive coping response (Mostalac-Preciado et al., 2011; Wu et al., 2013). The reason for these conflicting results is unclear but may reflect the complexity of the stress adaptation response, promiscuity and/or systemic effects of the pharmacological agents, and/or compensatory actions of the remaining receptor in knockout mice. Our similar findings in the forced swim test for both the MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice suggests that hippocampal MR plays an important role in the transition of active to passive coping in female mice. A limitation to our studies is that knockout of GR and/or MR is not restricted to the hippocampus. We and others have shown that *Emx1*-cre mediated recombination occurs in other regions of the forebrain such as the cortex, basomedial amygdala, and basolateral amygdala (Oakley et al., 2021; Gorski et al., 2002; Xie et al., 2019). Receptor loss in these extra hippocampal regions may also contribute to the sex-specific effect on stress adaptation as well as the other behavioral phenotypes in the single and double knockout mice.

Sex differences in the male and female brain are well documented and attributed to differences in sex chromosome dosage and sex hormones (Dewing et al., 2003; Markham et al., 2003; Ocanas et al., 2022). The sex-specific effects that we observed in our male and female single and double knockout mice may reflect alterations in sex hormone signaling secondary to the hippocampal GR/MR imbalance. While our microarray data showed no changes in androgen receptor (AR) and estrogen receptor-beta (ER-beta) expression in any of the knockout mice, ER-alpha was upregulated in the hippocampus of female MR$^{Emx1-cre}$ mice (1.41-fold) and in both male (1.39-fold) and female (1.66-fold) GRMR$^{Emx1-cre}$ mice. In addition, the sexual dimorphic effects that accompany the GR/MR imbalance may also involve alterations in the recently described genomic cross talk between the sex hormone receptors and the glucocorticoid signaling pathway (Paakinaho and Palvimo, 2021). GR has been shown to alter the estrogen gene expression program by modulating ER-alpha recruitment to DNA responsive elements on target genes and/or by tethering to ER-alpha and modifying its association with transcriptional coregulators (Dhaibar et al., 2021; Miranda et al., 2013; Yang et al., 2017). GR has also been shown to influence the androgen transcriptome by occupying genomic sites normally bound by AR (Arora et al., 2013). The altered expression of ER in the female knockout mouse models in conjunction with its multifaceted genomic interaction with GR (and presumably MR) may account for the greater number of sex-specific gene changes in the female knockout hippocampi compared to their male counterparts.

Alterations in the GR/MR balance also had major sex independent effects in the hippocampus. In both male and female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice, the expression of the classic CA2 neuronal markers PCP4 and RGS14 was abolished. Our genome-wide microarray data also revealed dramatic reductions in the expression of other known CA2 neuronal markers in these mice such as Adora1, Amigo2, Calb1, Necab2, and Smoc2. These gene changes were due to the deficiency in MR rather than aberrant GR signaling since the GRMR$^{Emx1-cre}$ mice phenocopied

the MR$^{Emx1-cre}$ mice. CA2 neurons exhibit unique molecular, morphological, and physiological properties that distinguish them from neighboring CA1 and CA3 cells (Dudek et al., 2016; Evans et al., 2015; Robert et al., 2018). These neurons lack most forms of synaptic plasticity and are resistant to cell death, and recent studies suggest that they are important for social memory, aggression, and novelty detection (Alexander et al., 2016; Hitti and Siegelbaum, 2014; Leroy et al., 2018). The altered molecular profile of CA2 neurons following MR deletion may lead to aberrant functional properties of these neurons. Indeed, CA2 neurons gain the ability to undergo long-term potentiation in mice lacking MR in the whole brain (McCann et al., 2021). The molecular and functional changes in CA2 neurons triggered by MR alterations may have important implications for understanding and treating stress-related psychiatric disorders, such as schizophrenia, that have been linked to CA2 dysregulation (Chevaleyre and Piskorowski, 2016).

Both male and female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ mice showed sex independent reductions in anxiety-like behavior in the elevated plus maze test. Since our GRMR$^{Emx1-cre}$ mice phenocopied the single MR$^{Emx1-cre}$ mice, our study demonstrates that the deficiency in MR rather than aberrant GR signaling is the primary driver of the anxiolytic effect. Analysis of the transcriptomic data from our knockout mouse models revealed 7 genes commonly altered in both the male and female MR$^{Emx1-cre}$ and GRMR$^{Emx1-cre}$ hippocampi that are associated with anxiety and are potential target genes of MR. One of these gene changes was a marked decrease in FK506-binding protein 5 (Fkbp5) expression. This reduced expression profile of Fkbp5 was validated in an independent set of knockout mice (Fig. 8). Consistent with this decrease, recent studies have demonstrated that MR directly upregulates the expression of Fkbp5 in the hippocampus of mice at baseline when corticosterone levels are low (Hartmann et al., 2021). Reductions in the levels or activity of Fkbp5 in the mouse brain have an anxiolytic effect (Attwood et al., 2011; Hartmann et al., 2015), and increased expression of Fkbp5 in humans has been associated with increased risk of developing stress related anxiety disorders (Darby et al., 2016; Klengel et al., 2013). How Fkbp5 dysregulation influences anxiety-like behavior is unclear. Fkbp5 has been shown to function in coordination with heat shock protein 90 to inhibit GR activity (Criado-Marrero et al., 2018). However, since the same anxiolytic behavior occurs in the double knockout mice, other actions of Fkbp5 are likely to be involved such as its ability to modulate ion homeostasis through interactions with transmembrane channels (Criado-Marrero et al., 2018).

Imbalances in hippocampal GR and MR signaling also had sex independent effects on neurodegeneration. The male and female GRMR$^{Emx1-cre}$ mice exhibited extensive neuronal loss in the dentate gyrus between 3 and 4 months of age. The concurrent loss of these two receptors also had a much greater impact on the hippocampal transcriptome than depletion of either receptor alone, suggesting that many genes depend on the joint actions of GR and MR for their appropriate expression. Analysis of the transcriptomic data identified 75 GR/MR target genes commonly altered in the male and female GRMR$^{Emx1-cre}$ hippocampus that were strongly associated with cell death and cell proliferation processes shown previously to be involved in the neurodegeneration phenotype (Oakley et al., 2021). Interestingly, these genes included 2 members of the Kruppel-like factor family of transcription factors, Klf9 and Klf13. Klf9 and Klf13 are each known to be directly upregulated by glucocorticoids in the hippocampus, and both hormone-activated GR and MR are recruited to the same DNA responsive elements in proximity to these genes (Kennedy et al., 2023; Mifsud et al., 2021). Consistent with this joint GR and MR regulation, the expression of both Klf9 and Klf13 was decreased only in the GRMR$^{Emx1-cre}$ hippocampus lacking the two receptors. The reduced expression profile of Klf9 was validated in an independent set of knockout mice (Fig. 10). Klf9 is critical for the later stages of neurogenesis in the dentate gyrus and for the survival of adult born dentate gyrus granule neurons (Scobie et al., 2009), and Klf13 has cytoprotective actions in hippocampal neurons (Avila-Mendoza et al., 2020). Therefore, the reduced expression of these 2 genes may contribute to the impaired neurogenesis and enhanced neuronal cell death underlying the neurodegeneration phenotype in the male and female GRMR$^{Emx1-cre}$ hippocampus. By maintaining appropriate expression levels of Klf9 and Klf13, GR and MR work together in a sex independent manner to support proper neuronal homeostasis.

5. Conclusion

In conclusion, using mice with conditional knockout of GR, MR, or both GR and MR in the hippocampus, we show that imbalances in GR and MR signaling have both sex-dependent and independent effects on the genetic, molecular, and functional properties of the hippocampus. These findings may inform new strategies for treating men and women with stress-related neuropsychiatric disorders.

Data statement

The authors declare that all supporting data are available within the article, or from the corresponding author, upon reasonable request.

CRediT authorship contribution statement

Robert H. Oakley: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Natallia V. Riddick:** Methodology, Investigation. **Sheryl S. Moy:** Methodology, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **John A. Cidlowski:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2023.100589.

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