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Glucocorticoid Receptor Translational Isoforms Generate Unique Glucocorticoid Responses in the Mouse Brain

Robert H. Oakley[1] | Tatsuya Sueyoshi[1] | Sivapriya Ramamoorthy[1] | Viktoriya D. Nikolova[2] | David R. Goulding[3] | Jianying Li[4] | Manas K. Ray[5] | Sheryl S. Moy[2] | John A. Cidlowski[1]

[1]Molecular and Cellular Biology Laboratory, NIEHS, NIH, DHHS, Research Triangle Park, North Carolina, USA | [2]Department of Psychiatry and Carolina Institute for Developmental Disabilities, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA | [3]Comparative Medicine Branch, NIEHS, NIH, DHHS, Research Triangle Park, North Carolina, USA | [4]Biostatistics and Computational Biology Branch, NIEHS, NIH, DHHS, Research Triangle Park, North Carolina, USA | [5]Gene Editing and Mouse Model Core, NIEHS, NIH, DHHS, Research Triangle Park, North Carolina, USA

Correspondence: John A. Cidlowski (cidlows1@niehs.nih.gov)

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ABSTRACT

Glucocorticoids are primary stress hormones necessary for life that act on nearly every tissue in the body to maintain homeostasis. These hormones and their synthetic derivatives are widely used in the clinic to combat disease but are limited by serious adverse effects. The actions of glucocorticoids are mediated by the glucocorticoid receptor (GR). In addition to the classic full-length receptor (GR-A), seven highly conserved receptor isoforms with progressively shorter N-terminal transactivation domains (NTD) (GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, GR-D3) are produced from the single GR gene by alternative translation initiation. To investigate the physiological function of these isoforms, we developed knockin mice that express GR-A but lack the GR NTD translational isoforms. Analyses of the hippocampal transcriptome from wild-type and GR-A knockin mice treated with dexamethasone (Dex) revealed three classes of Dex-regulated genes: genes dependent on GR-A alone, genes dependent on the GR NTD translational isoforms, and genes dependent on GR-A only under conditions of GR NTD isoform deficiency. The genes dependent on the GR NTD isoforms were preferentially associated with circadian rhythm signaling, synaptic function, and cognition. Consistent with these gene enrichment results, the GR-A knockin mice exhibited alterations in hypothalamic–pituitary–adrenal axis activity and fear-motivated contextual learning. The GR NTD translational isoforms formed distinct molecular complexes with GR-A and with each other, providing a mechanistic basis for their unique transcriptional signatures. These findings demonstrate that the GR NTD translational isoforms contribute to the actions of glucocorticoids in the brain by generating heterogeneity in glucocorticoid signaling.

1 | Introduction

Glucocorticoids are synthesized and released by the adrenal glands following activation of the hypothalamic–pituitary–adrenal (HPA) axis in a circadian manner and in response to physical and emotional stress [1]. These hormones act on nearly all cells and tissues, and they function to maintain homeostasis. Numerous physiological processes are regulated by glucocorticoids, including immune function, metabolism, cognition, behavior, skeletal growth, and cardiovascular function [2]. The powerful immunosuppressive actions of glucocorticoids have made synthetic derivatives of these steroids one of the most prescribed drug classes in the world today for treating inflammation, autoimmune disorders, cancer, and organ transplant rejection [3]. Unfortunately, the therapeutic benefit of glucocorticoids is limited by the development of side effects such as growth retardation in children, osteoporosis, hypertension, diabetes, abdominal obesity, anxiety, depression, and cognitive impairments. Understanding the molecular factors that control how cells and tissues respond to glucocorticoids is crucial for the development of new glucocorticoids and/or treatment regimens with improved health benefit/risk ratios.

The physiological and pharmacological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors [4]. Like other members of this family, GR is a modular protein comprised of an N-terminal transactivation domain (NTD), a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The single GR gene (*Nr3c1*) gives rise to the classic full-length receptor (GR-A) and 7 additional isoforms with progressively shorter NTDs (GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, and GR-D3) via highly conserved alternative translation initiation sites (Figure 1A) [1, 5–7]. The 7 GR NTD translational isoforms have an intact DBD and LBD, and these receptor subtypes bind glucocorticoids and DNA in a manner similar to GR-A [7]. However, the role played by this array of receptor isoforms in glucocorticoid physiology and pathophysiology is poorly understood. Genome-wide transcriptomic approaches performed in cultured cells have revealed that the GR NTD translational isoforms have gene regulatory profiles that differ from GR-A on some genes but not others [7–9]. Consistent with this finding, mice expressing only the GR-C3 isoform were deficient in their ability to repress a large cohort of immune and inflammatory response genes [8]. The GR NTD translational isoforms have also been shown to exhibit tissue-specific differences in expression. In rodents, the GR-C isoforms are most abundant in the pancreas and colon whereas the GR-D isoforms are highest in the spleen and lung [6]. The human placenta displays alterations in the expression profile of the GR subtypes based on several factors including gestational age, fetal sex, and maternal alcohol consumption [10, 11]. Furthermore, the complement of GR translational isoforms changes in the human brain during development, in the aging process, and in certain disease states such as bipolar disorder and schizophrenia [12–14]. These studies suggest the GR NTD translational isoforms may contribute to the distinct glucocorticoid responses seen in different cells and tissues.

To investigate the physiological function of the GR NTD translational isoforms, we developed mice that express GR-A but not the GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, and GR-D3 isoforms. The GR-A knockin mice showed no overt developmental, growth, or survival abnormalities. Given the important role glucocorticoids play in the brain and that alterations in hippocampal glucocorticoid signaling have been associated with many stress-related neuropsychiatric and learning disorders, we examined the glucocorticoid transcriptome in the hippocampus of wild-type (WT) and GR-A knockin mice that were treated with the synthetic glucocorticoid dexamethasone (Dex). Three different classes of Dex-regulated genes were identified: genes requiring only GR-A for their Dex regulation, genes requiring the expression of the GR NTD translational isoforms for their Dex regulation, and genes requiring GR-A and a concomitant deficiency in the GR translation isoforms for their Dex regulation. Gene enrichment annotations related to circadian rhythm, synapse morphology and function, and learning and memory were strongly associated with the genes dependent on the GR NTD translational isoforms for their regulation by glucocorticoids. In accord with these gene enrichment predictions, the GR-A knockin mice displayed increased corticosterone levels following acute stress and enhanced contextual learning in a conditioned fear test. Marked differences were found in the capacity of the GR translational isoforms to form complexes with GR-A and with each other, providing a mechanistic basis for their unique influence on the glucocorticoid transcriptome. These findings demonstrate that the GR NTD translational isoforms play a crucial role in the hippocampal response to glucocorticoids by generating heterogeneity in glucocorticoid signaling profiles.

2 | Materials and Methods

2.1 | Generation of GR-A Knockin Mice

Embryonic stem (ES) cells in which exon 2 of the *Nr3c1* (GR) gene was replaced by a Neo cassette flanked by an upstream lox71 site and downstream lox2272 site were generated by standard gene targeting procedures and have been previously described [8]. An exchange vector was generated that contained exon 2 of the GR gene in which the ATG (methionine) start codons for the translational isoforms GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, and GR-D3 were mutated to ATC (isoleucine). The GR-A start codon was left intact. The point mutations were performed using the QuikChange kit (Stratagene) and confirmed by DNA sequencing. The exchange vector also contained a lox66 site followed by a hygromycin cassette flanked by Frt sites that was positioned upstream of the mutated exon 2. A lox2272 site was positioned downstream of the mutated exon 2. The exchange vector and Cre-recombinase were transfected into the targeted ES cells by electroporation, and positive cells were selected using G418 (sensitive) and hygromycin (resistant). ES cells undergoing successful cassette exchange were confirmed by PCR. The GR-A positive ES cell clone was injected into C57BL/6 blastocysts to create chimeric mice. Chimeric males were bred with C57BL/6 females for germline transmission, and the hygromycin cassette was deleted by crossing mice with Flp-deleter (C57BL/6-Tg(CAC-Flpe)2Arte) (Taconic Biosciences, #7089, RRID:IMSR_TAC:7089) mice that had been backcrossed 7 generations to C57BL/6J mice (The Jackson Laboratory, #000664, RRID:IMSR_JAX:000664). The resulting $Nr3c1^{WT/GRA}$ mice were maintained on a C57BL/6 background and intercrossed to produce $Nr3c1^{WT/WT}$ (wild-type or WT), $Nr3c1^{WT/GRA}$ (heterozygous GR-A knockin), and $Nr3c1^{GRA/GRA}$ (homozygous GR-A knockin) mice. The mutated translational start codons and intact GR-A start codon in exon 2 were also confirmed by DNA sequencing of amplified genomic DNA from GR-A knockin mice. Mice were housed on a 12 h:12 h light/dark cycle with access to food and water ad libitum. Mouse genotypes were determined at weaning from ear biopsies using real time PCR with specific probes designed for the GR gene (Transnetyx, Cordova, TN). Data presented are from adult male mice. All experiments 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).

2.2 | Immunoblot Analysis

Tissues were removed from WT and GR-A knockin mice approximately 2–4 months of age and lysed in RIPA buffer

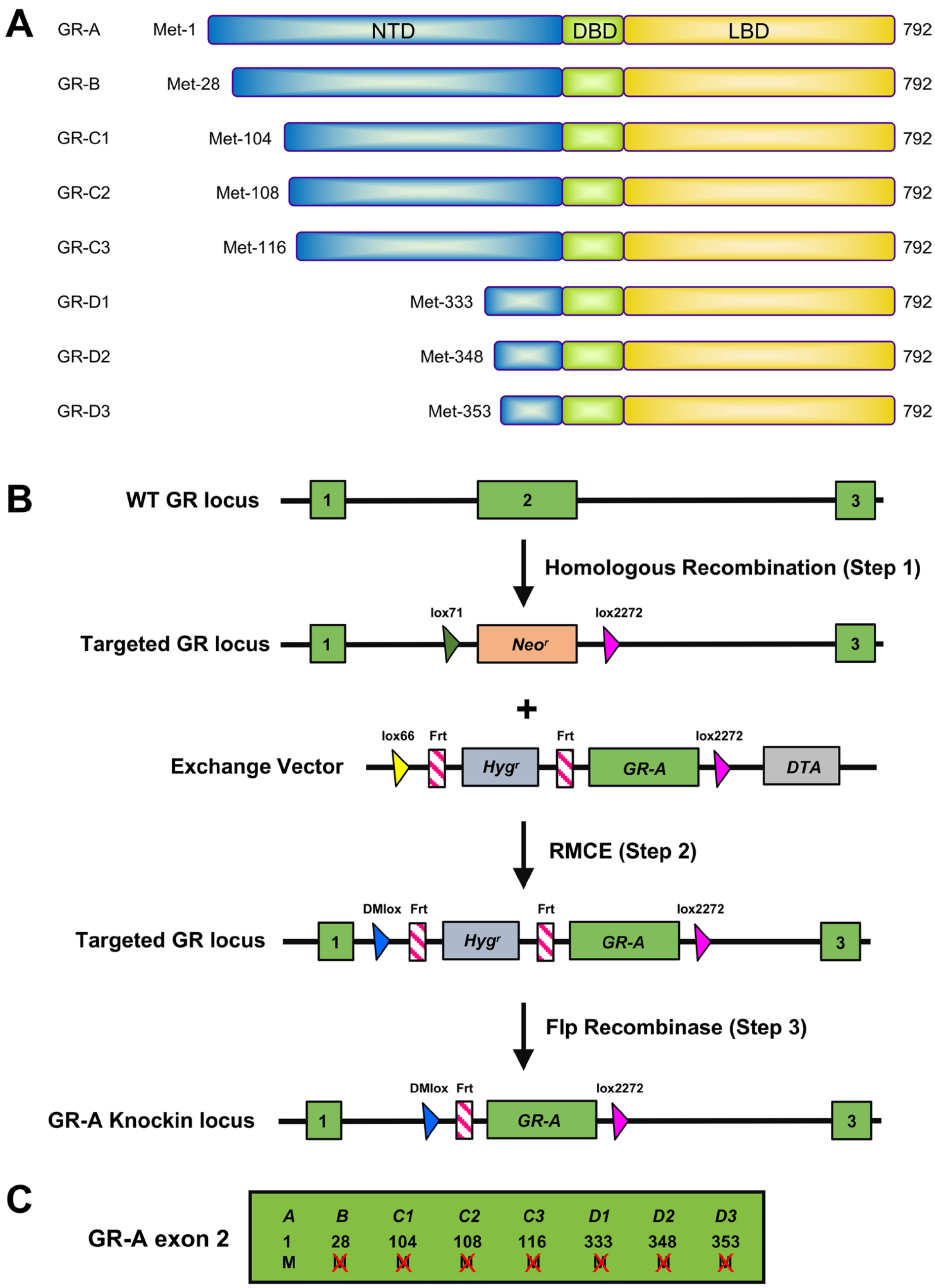

FIGURE 1 | Generation of the GR-A knockin mice. (A) Schematic showing the classic full-length GR-A isoform and the 7 GR NTD translational isoforms GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, and GR-D3. (B) Three major steps were involved in the development of GR-A knockin mice. In step 1, ES cells were generated by homologous recombination in which exon 2 of the GR gene was replaced with a Neo^r selection cassette flanked by lox71 and lox2272 sites. In step 2, the targeted ES cells were transfected with Cre and an exchange vector containing a mutated version of GR exon 2 that permits expression of GR-A but not the 7 translational isoforms. The exchange vector also contained a Hyg^r selection marker flanked by Frt sites. Flanking the Hyg^r and mutant GR exon 2 cassettes were lox66 and lox2272 sites that permit Cre-dependent directional insertion of DNA in conjunction with the lox71 and lox2272 sites on the targeted allele. Cre recombinase mediated cassette exchange (RMCE) at the lox71 and lox66 sites generates a double mutant lox site (DMlox). In step 3, the GR-A positive ES cells were injected into blastocysts derived from C57BL/6 mice. Chimeric males were bred with C57BL/6 females for germline transmission, and the resulting GR-A knockin progeny were bred with Flp deleter mice to remove the Hyg^r cassette. (C) Schematic showing mutated GR exon 2. The ATG start codons for GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, and GR-D3 were mutated to ATC. The ATG start codon for GR-A was left intact. The initiator methionine (M) amino acid number is depicted for each of the translational variants.

(ThermoFisher Scientific #89901) with protease inhibitors (Millipore-Sigma #11836153001). HEK293 cells expressing the GR isoform fusions with the Nanobit tags (LgBit or SmBit) were lysed in sodium dodecyl sulfate sample buffer. Nitrocellulose membranes with equivalent amounts of protein were analyzed and quantitated using the Odyssey Infrared Imaging System (LI-COR Biosciences). Blots were incubated with anti-GR (Cell Signaling, #3660, RRID:AB_11179215) and anti-actin

(Millipore, #MAB1501, RRID:AB_2223041) primary antibodies followed by goat anti-rabbit Alexa Fluor 680-conjugated (Life Technologies, #A21109, RRID:AB_2535758) and goat anti-mouse IRDye800-conjugated (LI-COR Biosciences, #926-32210, RRID:AB_621842) secondary antibodies.

2.3 | RNA-Seq Analysis

WT and GR-A knockin mice were adrenalectomized (ADX) at approximately 2.5 months of age to remove endogenous glucocorticoids. Two weeks after surgery, the ADX WT and GR-A knockin mice were injected intraperitoneally with PBS or 1 mg/kg Dex ($n = 5$ mice per genotype and treatment group). After a 6-h treatment, mice were euthanized, and total RNA was isolated from the hippocampus using Qiagen RNeasy Mini kit. A TruSeq mRNA stranded kit (Illumina, San Diego, CA) was utilized to prepare the poly(A)-enriched RNA-seq libraries that were sequenced on the Illumina NovaSeq 6000 in a 75-base paired-end mode according to the manufacturer's protocol. The quality of raw RNA-seq sequences for each sample was assessed with FastQC (v.0.11.5). Reads (average of 30 million per sample) were mapped to the University of California Santa Cruz (UCSC) mm39 reference genome using STAR (v.2.6.0c, RRID:SCR_004463). The quantification results from featureCounts (v.2.0.6) were accomplished based on Gencode vM34 as the gene model, and the final counts matrix of known genes was analyzed with the Bioconductor package DESeq2 (v.1.38.3, RRID:SCR_006442), which fits a negative binomial distribution to estimate technical and biological variability. A factorial design was implemented to capture genomic knockin manipulation and treatment effects, and differentially expressed genes were obtained from the individual contrasts using the Benjamini-Hochberg adjusted p-value (q-value) < 0.05. All significant differentially expressed genes were used in downstream analyses without application of a fold change cut-off. Other statistical analyses were conducted in R with customized programs. Ingenuity pathway analysis (IPA) (RRID:SCR_008653) was performed on the differentially expressed genes to determine functional enrichment. The RNA-seq data has been deposited in the Gene Expression Omnibus with the accession number (GSE298763).

2.4 | Real-Time PCR (RTPCR)

For validation of the RNA-seq, an independent set of ADX WT and GR-A knockin mice were injected intraperitoneally with PBS or 1 mg/kg Dex for 6 h. The hippocampus was removed, and total RNA was isolated using the RNeasy Mini and RNase-Free DNase kits (Qiagen). Selection criteria for the 8 genes chosen for validation included strong q-values, a range in fold change values, and both up- and down-regulation profiles. Individual mRNA abundance was determined using a TaqMan one-step RTPCR procedure on the CFX Duet RTPCR sequence detection system (BioRad). All primer/probe sets were purchased from ThermoFisher Scientific. Relative expression values for each gene were calculated using the double delta Ct analysis method and the housekeeping gene peptidyl-prolyl isomerase B (*Ppib*).

2.5 | Corticosterone Measurements

Mandibular bleeds were performed on the same set of WT and GR-A knockin mice approximately 3–5 months of age on three separate occasions: in the morning (8:00 a.m. to 9:00 a.m.), evening (7:00 p.m. to 8:00 p.m.), and in the morning (7:30 a.m. to 9:30 a.m.) immediately following restraint stress. Two weeks intervened between the different bleeds. For restraint stress, mice were placed in ventilated holders (Kent Scientific) for 30 min. Mandibular bleeds were also performed on ADX mice approximately 10 days after the surgery between 11:45 a.m. and 1:00 p.m. Serum corticosterone levels were measured using an Enzyme Immunoassay Kit (Arbor Assays, Ann Arbor, MI).

2.6 | 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 on male WT ($n = 13$) and GR-A knockin ($n = 14$) mice using published methods [15, 16]. The same group of male mice underwent all behavioral tests. Mice were approximately 7 weeks in age at the beginning of the behavioral testing regimen, with assays carried out across 9 weeks in the following order: elevated plus maze, open field, accelerating rotarod, acoustic startle, and conditioned fear.

2.7 | Elevated Plus Maze Test

WT and GR-A knockin mice were given one 5-min trial on the 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. The time spent on the open arms and the number of entries into the open arms are indices of anxiety-like behavior.

2.8 | Open Field Test

Exploratory activity in a novel environment was assessed in WT and GR-A knockin mice by a one-hour trial in an open field chamber crossed by a grid of photobeams (VersaMax system, AccuScan Instruments). Counts were taken of the number of photobeams broken during the trial in 5-min intervals, with measures taken of locomotion (total distance traveled), rearing movements, and time spent in the center region of the open field, an index of anxiety-like behavior.

2.9 | Accelerating Rotarod Test

WT and GR-A knockin were tested for motor coordination and learning on an accelerating rotarod (Ugo Basile, Stoelting Co., Wood Dale, IL). For the first test session, mice were given three trials, with 45 s between each trial. Two additional trials were given 48 h later. Rpm (revolutions per minute) was set at an initial value of 3, with a progressive increase to a maximum of 30 rpm across 5 min (the maximum trial length). Measures were taken for latency to fall from the top of the rotating barrel.

2.10 | Acoustic Startle Test

WT and GR-A knockin mice were tested for auditory function, reactivity to environmental stimuli, and sensorimotor gating using the acoustic startle test. Mice were placed into individual small Plexiglas cylinders within larger, sound-attenuating chambers. Each cylinder was seated upon a piezoelectric transducer, which allowed vibrations to be quantified and displayed on a computer (San Diego Instruments SR-Lab system). The chambers included a ceiling light, fan, and a loudspeaker for the acoustic stimuli. Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter (San Diego Instruments). Each session consisted of 42 trials that began with a 5-min habituation period. There were 7 different types of trials: the no-stimulus trials, trials with the acoustic startle stimulus (40 ms; 120 dB) alone, and trials in which a prepulse stimulus (20 ms; either 74, 78, 82, 86, or 90 dB) occurred 100 ms before the onset of the startle stimulus. Measures were taken of the startle amplitude for each trial across a 65-ms sampling window, and an overall analysis was performed for each subject's data for levels of prepulse inhibition at each prepulse sound level [calculated as 100 − (response amplitude for prepulse stimulus and startle stimulus together/response amplitude for startle stimulus alone × 100)].

2.11 | Conditioned Fear Test

WT and GR-A knockin mice were evaluated for learning and memory in a conditioned fear test (Near-Infrared image tracking system, MED Associates, Burlington, VT). The procedure had the following phases: training on Day 1, a test for context-dependent learning on Day 2, and a test for cue-dependent learning on Day 3. For training on Day 1, each mouse was placed in the test chamber, allowed to explore for 2 min, and then exposed to a 30-s tone (80 dB) that co-terminated with a 2-s scrambled foot shock (0.4 mA). Mice received 2 additional shock-tone pairings, also termed conditioned stimulus-unconditioned stimulus (CS-US) presentations, with 80 s between each pairing. On Day 2, mice were placed back into the original conditioning chamber for a test of contextual learning. Levels of freezing (an index of learning) were determined across a 5-min session. On Day 3, mice were placed in a modified conditioning chamber for a test of cue learning. The conditioning chambers were modified using a Plexiglas insert to change the wall and floor surface, and a novel odor (dilute vanilla flavoring) was added to the sound-attenuating box. Mice were placed in the modified chamber and allowed to explore. 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 session.

2.12 | Nanobit Protein–Protein Interaction Assay

The Nanobit assay was performed as previously described [17]. GR-A, GR-B, GR-C3, and GR-D3 human cDNAs were cloned into pBiT1.1-C[TK/LgBiT] and pBit2.1-C[TK/SmBiT] vectors (Promega) for C-terminal LgBit or SmBit fusion proteins. Fifty thousand HEK293 cells (RRID:CVCL_0045) were seeded into each well of white 96 well plates for NanoLuc luciferase activity detection using phenol red free OptiMEM media (Invitrogen) supplemented with 10% fetal bovine serum and penicillin–streptomycin. The cells were transfected with various combinations of the GR isoform vectors 24 h after seeding using Lipofectamine 3000 (Invitrogen). After a 48-h incubation, Nanobit luciferase activities in living cells were monitored for a total of 65 min, quantifying every 1 min using Nano-Glo live cell assay system (Promega) and GloMax Discover luminometer (Promega). Dex (100 nM final concentration) was added 25 min after the luciferase activity measurement had started.

2.13 | Statistical Analysis

An unpaired two-tailed *t*-test, one-way ANOVA with Tukey's multiple comparisons post hoc test, two-way ANOVA with Sidak's multiple comparisons post hoc test, or two-way repeated measures ANOVA with Sidak's or Tukey's multiple comparisons post hoc test were used to evaluate statistical significance (defined as $p < 0.05$). For behavioral studies, post hoc comparisons were conducted using Fisher's Protected Least Significant Difference (PLSD) tests only when a significant F value was found in the two-way repeated measures ANOVA. The Mantel-Cox log-rank test was used for survival curves. Data distribution was assumed to be normal, but this was not formally tested. The statistical analyses were performed using GraphPad Prism software (v.10.0.0; RRID:SCR_002798, Dotmatics, Boston, MA) and Statview (RRID:SCR_017411, SAS, Cary, NC).

3 | Results

3.1 | Development and Characterization of GR-A Knockin Mice

To elucidate the physiological function of the GR NTD translational isoforms, we used a knockin approach to develop mice that express the classic full-length GR (GR-A) but lack all 7 translational subtypes (Figure 1A). The GR-A knockin mice were generated by replacing exon 2 of the GR gene with a modified exon 2 in which the GR-A ATG start codon was left intact but the GR-B, GR-C1, GR-C2, GR-C3, GR-D1, GR-D2, and GR-D3 ATG start codons were mutated to ATC (isoleucine) (Figure 1B,C). The GR-A knockin mice were born at the expected Mendelian frequency and

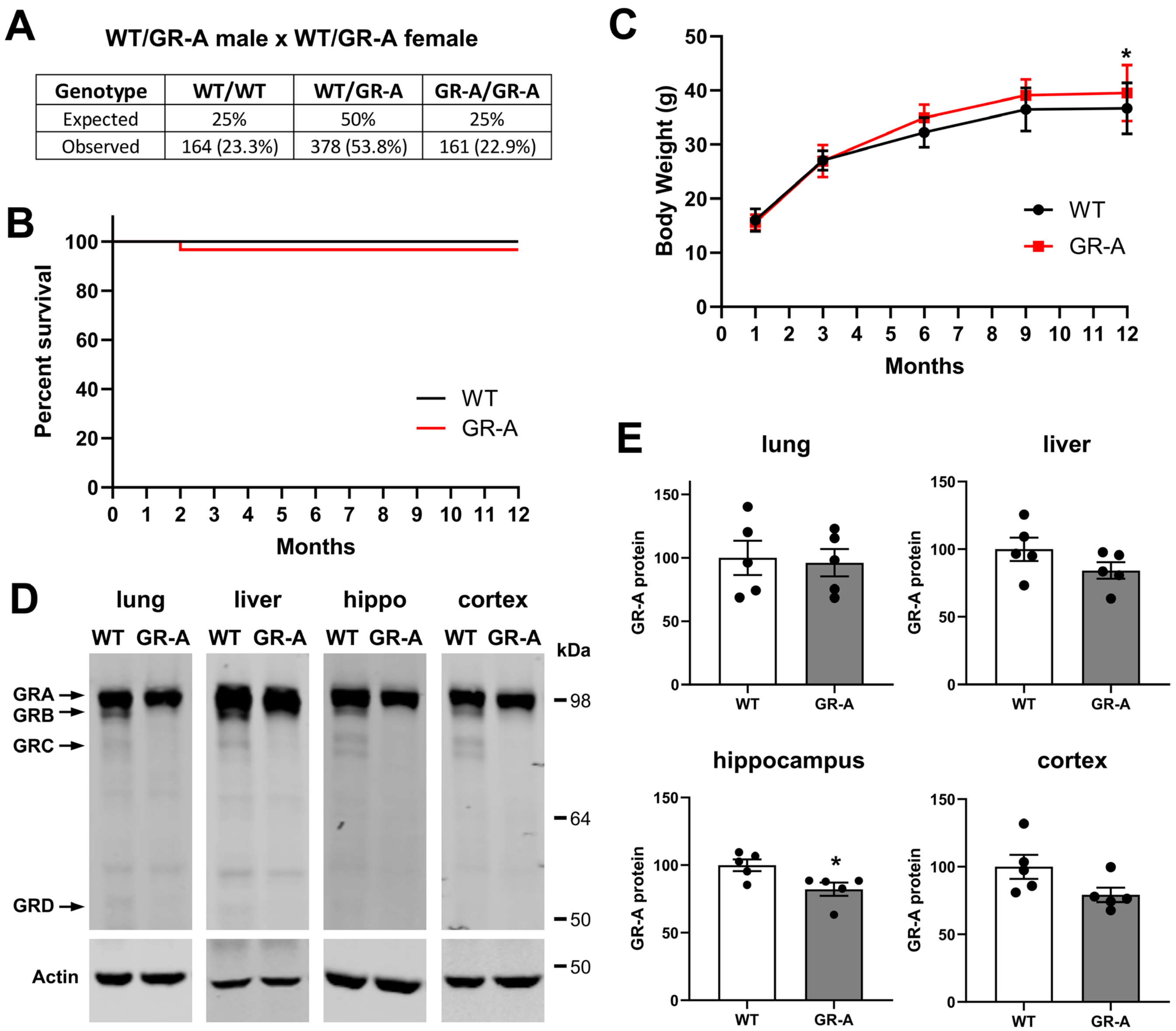

FIGURE 2 | Characterization of GR-A knockin mice. (A) Expected and observed genotypes from intercross of heterozygous GR-A knockin mice ($n = 703$ mice). (B) Survival curves for WT and GR-A knockin mice ($n = 27$–30 mice per genotype). Mantel-Cox log-rank test revealed no significant differences between genotypes. (C) Body weights were determined for WT and GR-A knockin mice at 1, 3, 6, 9, and 12 months of age. Data are mean ± SD ($n = 7$–29 mice per genotype). Two-way ANOVAs revealed main effects of time [$F(4,132) = 126.0$, $p < 0.0001$] and genotype [$F(1,132) = 4.661$, $p = 0.0327$] on body weight. Sidak's multiple comparison post hoc test was performed to determine significance at each time point. *$p < 0.05$ for GR-A compared to WT. (D) Representative immunoblots showing GR-A, GR-B, GR-C, and GR-D isoforms from the lung, liver, hippocampus, and cortex of WT and GR-A knockin mice. (E) Quantitation of GR-A isoform levels from immunoblots of WT and GR-A knockin lung, liver, hippocampus, and cortex lysates. Data are mean ± SEM ($n = 5$ mice per genotype). A *t*-test was performed to determine significance. *$p < 0.05$ for GR-A compared to WT.

exhibited growth and survival curves very similar to WT mice (Figure 2A–C). A small increase in body weight was observed at the 12-month time point for the GR-A knockin mice. We examined GR expression levels in protein lysates prepared from the lung, liver, hippocampus, and cortex of adult WT and GR-A knockin mice (Figure 2D). In the WT mice, immunoblot analysis demonstrated abundant expression of the GR-A isoform in each tissue. The GR-B, GR-C, and GR-D translational isoforms were also detected in each WT tissue. A longer exposure was required to observe the GR-D subtypes suggesting these isoforms are expressed at low levels in these tissues (Figure S1). In the GR-A knockin mice, the GR-A isoform was expressed at levels comparable to that measured in the WT tissues with a small reduction being detected in the hippocampus (Figure 2D,E). The expression of the GR-B, GR-C and GR-D translational isoforms was eliminated in each of the GR-A knockin mouse tissues (Figure 2D; and Figure S1). These data demonstrate that we have successfully developed mice that express the GR-A isoform but lack the 7 GR NTD translational isoforms.

3.2 | Glucocorticoid Transcriptome in the Hippocampus of WT and GR-A Knockin Mice

To investigate the in vivo contribution of the 7 GR NTD translational isoforms to the glucocorticoid signaling profile, we performed an RNA-seq on RNA isolated from the hippocampus of adult male adrenalectomized (ADX) WT and GR-A knockin mice that were treated for 6 h with PBS or Dex. The hippocampus was chosen because it is a classic glucocorticoid target tissue and alterations in glucocorticoid signaling in the hippocampus have been associated with many stress-related neuropsychiatric disorders and cognitive impairments. Corticosterone levels were negligible in the ADX WT and GR-A knockin mice and did not differ between treatment groups nor genotypes (Figure 3A). A total of 1770 genes were regulated by Dex in the WT hippocampus and 1941 genes were regulated by Dex in the GR-A knockin hippocampus (Figure 3B; and Figure S2). The proportion of genes induced (57.2% in WT; 51.9% in GR-A) and repressed (42.8% in WT; 48.1% in GR-A) by Dex was similar in the 2 genotypes. Venn diagram analysis showed that the majority of the Dex-regulated genes in the WT hippocampus (1305 of 1770 genes, or 73.7%) were commonly regulated by Dex in the GR-A knockin hippocampus (Figure 3C). The polarity of the Dex response was the same for each of the 1305 common genes, and the magnitude of the Dex response was similar as revealed by the fitted line slope of approximately 1.0 when comparing the WT versus GR-A fold change levels (Figure 3D). This common gene regulatory profile in both animal models suggests the GR-A isoform can regulate the transcription of these 1305 hippocampal genes independent of the GR NTD translational isoforms. These genes, therefore, depend on GR-A alone (Figure 3C and Table S1). The Venn diagram analysis also revealed two additional sets of Dex-regulated genes. First was a set of 465 genes that were regulated by Dex in the WT hippocampus but not the GR-A hippocampus. The unique regulation of these genes by Dex only in the WT hippocampus indicates that the expression of the GR NTD translational isoforms is required for certain genes to be regulated by glucocorticoids. These genes, therefore, depend on the GR NTD isoforms (Figure 3C and Table S2). Second was the set of 636 genes that were regulated by Dex in the GR-A hippocampus but not the WT hippocampus. The unique regulation of these genes by Dex only in the GR-A knockin hippocampus indicates that the GR-A isoform gains the ability to regulate certain genes under conditions of GR NTD translational isoform deficiency. These genes, therefore, depend on GR-A under conditions of GR NTD isoform deficiency (Figure 3C and Table S3).

To validate the WT unique 465, WT/GR-A common 1305, and GR-A unique 636 sets of Dex-regulated genes identified from the RNA-seq, we performed RTPCR on representative members of each gene group from an independent set of ADX WT and GR-A knockin mice. From the WT unique 465 genes that depend on the expression of GR NTD translational isoforms for their Dex regulation, lipocalin 2 (*Lcn2*) mRNA was upregulated and syntrophin beta 1 (*Sntb1*) mRNA was downregulated by Dex only in the hippocampus of WT mice (Figure 3E). From the WT/GR-A common 1305 genes that depend on the GR-A isoform alone for their Dex regulation, perilipin 4 (*Plin4*), sulfotransferase family 1A member 1 (*Sult1a1*), and kirre-like nephrin family adhesion molecule 2 (*Kirrel2*) mRNA were upregulated and tenascin C (*Tnc*) mRNA was down-regulated by Dex in the hippocampus of both the WT and GR-A mice (Figure 3E). From the GR-A unique 636 genes that depend on GR-A under conditions of GR NTD translational isoform deficiency for their Dex regulation, cytochrome P450 family 4 subfamily F member 15 (*Cyp4f15*) and Cdk5 and Abl enzyme substrate 1 (*Cables1*) mRNA were upregulated by Dex treatment only in the hippocampus of the GR-A mice (Figure 3E). The RTPCR results are consistent with the RNA-seq findings and confirm the presence of 3 groups of Dex-regulated genes in the mouse hippocampus: genes that depend on the 7 GR NTD translational isoforms, genes that depend on the GR-A isoform alone, and genes that depend on GR-A under conditions of GR NTD translational isoform deficiency.

We performed Ingenuity pathway analysis (IPA) to elucidate the nervous system signaling pathways that were associated with the three distinct sets of Dex-regulated genes in the mouse hippocampus. The top 10 most significant pathways are shown in Figure 4A. The ephrin receptor and axonal guidance signaling pathways were commonly enriched for all three sets of genes, suggesting pathways important for neuronal migration are major targets of glucocorticoids and that GR-A and the GR NTD translation isoforms work together to regulate genes in the same pathway. The three gene sets also showed preferential associations with specific pathways. Circadian rhythm signaling and synaptic long-term depression (LTD) were strongly associated with the genes requiring the GR NTD translational isoforms for their glucocorticoid regulation. CREB and glutaminergic receptor signaling pathways were strongly enriched for the genes that depend on the GR-A isoform alone for their regulation by glucocorticoids. Neuroinflammation and neuregulin signaling pathways were strongly associated with the genes regulated by GR-A under conditions of GR NTD translational isoform deficiency. The specific Dex-regulated genes in each of these enriched pathways are provided in Table S4.

IPA was also utilized to investigate the biological functions associated with the three sets of Dex regulated genes in the mouse hippocampus (Figure 4B). Consistent with the known hippocampal actions of glucocorticoids to regulate neuron development and neurogenesis, nervous system development and function was one of the top biological functions associated with the WT unique 465 genes and the WT/GR-A common 1305 genes. This annotation was also significantly enriched for the GR-A unique 636 genes though it was outside the top 10 (Figure S3). Within the high-level nervous system development and function IPA category, specific annotations related to the synapse (such as dendritic spines and synaptic transmission) were heavily enriched for the WT unique 465 genes but not the WT/GR-A common 1305 nor GR-A unique 636 genes (Figure 5A). The biological function Behavior was also significantly associated with the WT unique 465 genes and the WT/GR-A common 1305 genes, consistent with the known hippocampal actions of glucocorticoids to regulate the stress response, cognition, and mood (Figure S3). Interestingly, Behavior was not an enriched function for the GR-A unique 636 genes (Figure S3). Within the high-level Behavior IPA category, specific annotations related to cognition, learning, memory, and circadian rhythm were heavily enriched for the WT unique 465 genes but not the WT/GR-A common 1305 genes (Figure 5B). Anxiety, however, was strongly associated with both of these gene sets (Figure 5B). Collectively, these gene enrichment results suggest that the genes dependent on the GR NTD translational isoforms for their glucocorticoid regulation preferentially target the synapse to modulate HPA axis activity and learning and memory processes.

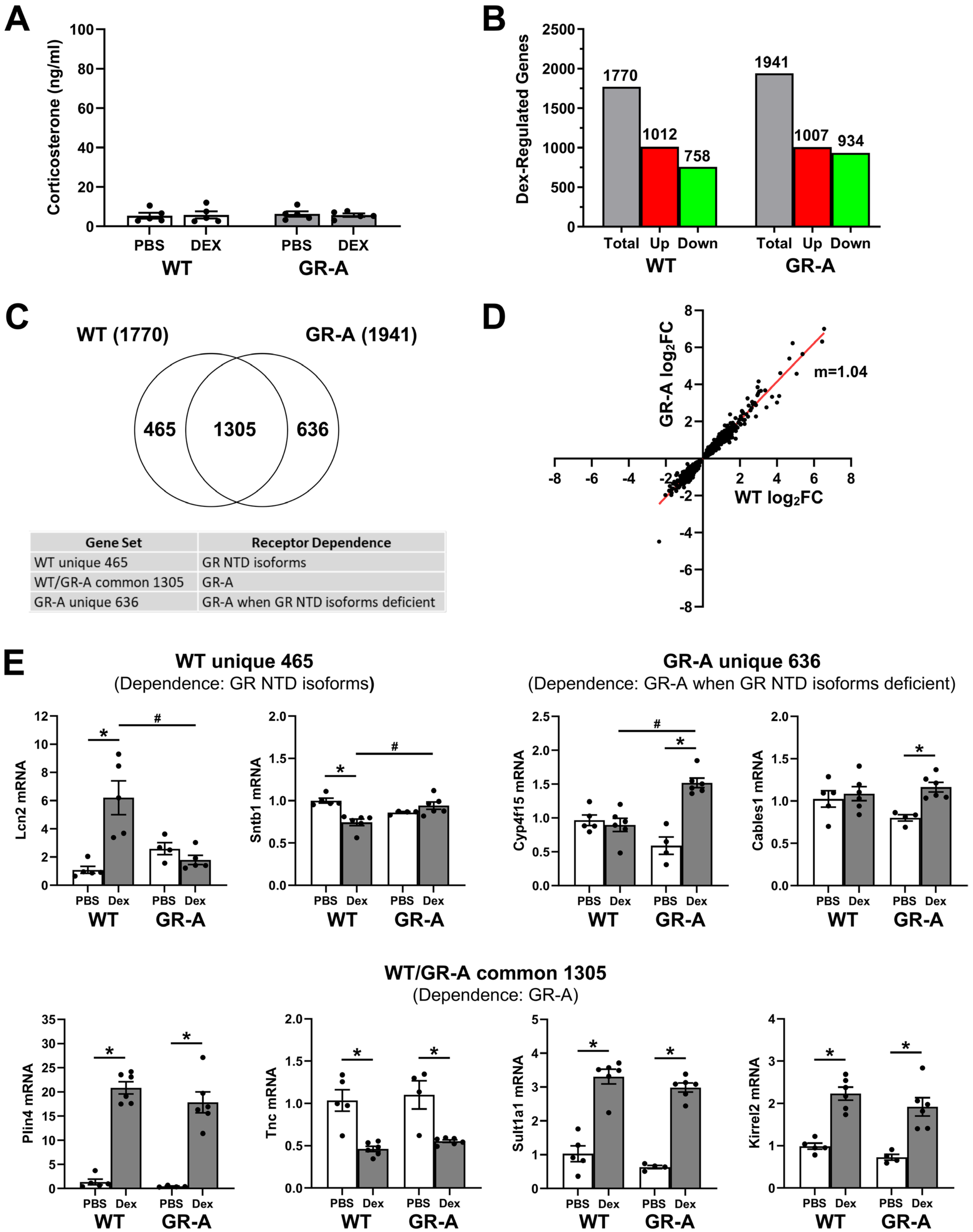

FIGURE 3 | Legend on next page.

FIGURE 3 | Glucocorticoid transcriptome in the hippocampus of WT and GR-A knockin mice. RNA-seq was performed on hippocampal RNA from ADX WT and GR-A knockin mice treated for 6 h with PBS or 1 mg/kg Dex. (A) Corticosterone levels measured at mid-day in ADX WT and GR-A knockin mice approximately 10 days after ADX surgery. Data are mean ± SEM ($n = 5$ mice per group). A one-way ANOVA revealed no significant differences. (B) Total number of genes regulated by Dex in the hippocampus of WT and GR-A knockin mice. Also shown are the number of genes induced (Up) or repressed (Down) by Dex. (C) Venn diagram comparison of the 1770 Dex-regulated genes in the WT hippocampus with the 1941 Dex-regulated genes in the GR-A knockin hippocampus. Also depicted is the GR dependence of each gene set. (D) RNA-seq data scatter plot of the WT/GR-A common 1305 genes showing their Dex regulation profile ($\log_2$-fold change) in the WT and GR-A knockin hippocampus. For each gene, the x-axis shows the Dex-mediated fold change in the WT hippocampus and the y-axis shows the Dex-mediated fold change in the GR-A knockin hippocampus. Also shown is the fitted straight line ($R^2 = 0.97$) and its slope ($m = 1.04$) from a nonlinear regression analysis of the data. (E) For validation of RNA-seq results, RTPCR was performed on hippocampal RNA isolated from an independent set of ADX WT and GR-A knockin mice treated with PBS or 1 mg/kg Dex for 6 h. Shown are lipocalin 2 (*Lcn2*) and syntrophin beta 1 (*Sntb1*) mRNA expression from the WT unique 465 gene set; perilipin 4 (*Plin4*), tenascin C (*Tnc*), sulfotransferase family 1A member 1 (*Sult1a1*), and kirre-like nephrin family adhesion molecule 2 (*Kirrel2*) mRNA expression from the WT/GR-A common 1305 gene set; and cytochrome P450 family 4 subfamily F member 15 (*Cyp4f15*) and Cdk5 and Abl enzyme substrate 1 (*Cables1*) mRNA expression from the GR-A unique 636 gene set. Data are mean ± SEM ($n = 4–6$ mice per group). One-way ANOVAs followed by Tukey post hoc tests were performed to determine significance. $^*p < 0.05$ for Dex compared to PBS within genotype. $^{\#}p < 0.05$ for Dex compared to Dex across genotypes.

3.3 | Altered Stress-Induced HPA Axis Activity and Fear-Motivated Contextual Learning in GR-A Knockin Mice

The gene enrichment analysis of the Dex regulated genes in the hippocampus suggested that the GR NTD translational isoforms may function to regulate HPA axis activity by targeting genes involved in circadian rhythm (Figures 4A and 5B). Therefore, we hypothesized that a deficiency in the glucocorticoid regulation of these genes in the GR-A knockin hippocampus may lead to a functional alteration in circadian and/or stress-induced corticosterone levels. To test this hypothesis, we first measured morning (circadian nadir) and evening (circadian peak) corticosterone levels in serum from adult WT and GR-A knockin mice (Figure 6A). Both the WT and GR-A knockin mice displayed an intact diurnal rhythm of corticosterone with significantly increased corticosterone levels in the evening compared to the morning time period. In addition, no differences were observed in the morning nadir or evening peak corticosterone levels between the WT and GR-A knockin mice. We next examined morning corticosterone levels following 30 min of restraint stress (Figure 6A). This acute stress produced a robust increase in corticosterone in both WT and GR-A knockin mice; however, the GR-A knockin mice exhibited a greater rise in the stress-induced levels of corticosterone than the WT mice. These findings suggest that the GR NTD translational isoforms play an important role in the regulation of HPA axis activity in response to stress.

The gene enrichment analysis of the glucocorticoid transcriptome in the hippocampus also implicated the GR NTD translational isoforms in the regulation of genes involved in learning and memory (Figure 5B). To determine if a deficiency in the glucocorticoid regulation of these genes in the GR-A knockin hippocampus altered cognition, we performed a conditioned fear test. For training on Day 1, mice were placed in a conditioning chamber, allowed to explore for 2 min, and then exposed to 3 shock-tone pairings. During the training session, neither the baseline levels of freezing nor the freezing response to the 3 tone-shock presentations differed between the WT and GR-A knockin mice (Figure 6B). In addition, no effects of genotype were found for activity levels during the foot shock (Figure 6C), suggesting the WT and GR-A knockin mice had similar sensitivity to the aversive stimulus. To investigate contextual learning, mice were placed back in the original conditioning chamber on Day 2 and levels of freezing were determined across a 5-min session (Figure 6D). The GR-A knockin mice exhibited a marked increase in the levels of freezing during the session compared to the WT mice, suggesting contextual learning was enhanced in mice lacking the GR NTD translational isoforms. In contrast, no alterations were observed for the GR-A knockin mice in cue-dependent learning, which was evaluated by placing the mice in a modified conditioning chamber on Day 3 and measuring levels of freezing before and during the acoustic stimulus (Figure 6E). The observed alterations in the fear-motivated contextual learning in the GR-A knockin mice could not be attributed to changes in anxiety-like behavior, locomotor activity, motor coordination, or auditory function, as these behavioral responses did not differ between the WT and GR-A knockin mice (Figures S4–S6). These results indicate that the GR NTD translational isoforms play a critical role in fear-motivated context-dependent learning.

3.4 | GR NTD Translational Isoforms Have Distinct Capacities to Form Homodimers and Heterodimers

Our findings in the hippocampus suggest that the GR NTD translational isoforms are required for the glucocorticoid dependent regulation of many genes. These NTD isoforms may directly regulate genes by themselves, or they may function in a cooperative manner with the GR-A isoform to elicit gene expression changes. A major factor controlling the transcriptional activity of the GR-A isoform is whether it functions as a monomer or homodimer [18–20]. Surprisingly, it is not known whether the GR NTD translational isoforms homodimerize. Moreover, the ability of the GR NTD translational isoforms to form heterodimeric complexes with themselves or with GR-A has not been previously explored. Differences in the capacity of the GR translational isoforms to interact with GR-A and with each other may underlie their ability to confer unique transcriptional responses to glucocorticoids. To investigate the ability of GR-A and GR NTD translational isoforms to associate in complexes, we used the Nanobit structural complementation luciferase

A

WT Unique 465
(Dependence: GR NTD isoforms)

Rank	Nervous System Signaling Pathways	p-value	z-score	Genes
1	Ephrin Receptor Signaling	1.17E-06	-0.632	15
2	Circadian Rhythm Signaling	5.25E-04	n/a	13
3	Opioid Signaling Pathway	7.08E-04	0.577	13
4	Axonal Guidance Signaling	8.13E-04	n/a	19
5	Ephrin B Signaling	1.15E-03	-0.816	6
6	Synaptic Long Term Depression	1.51E-03	-0.632	10
7	Serotonin Receptor Signaling	1.82E-03	-0.243	17
8	Synaptogenesis Signaling Pathway	2.04E-03	2.309	13
9	Dopamine-DARPP32 Feedback in cAMP Signaling	3.39E-03	1.134	9
10	GNRH Signaling	4.07E-03	-0.378	9

WT/GR-A Common 1305
(Dependence: GR-A)

Rank	Nervous System Signaling Pathways	p-value	z-score	Genes
1	Serotonin Receptor Signaling	1.58E-13	-0.384	62
2	Myelination Signaling Pathway	7.94E-12	0.59	47
3	Axonal Guidance Signaling	3.16E-11	n/a	61
4	CREB Signaling in Neurons	1.12E-10	-0.254	67
5	Docosahexaenoic Acid (DHA) Signaling	2.14E-09	1.372	36
6	WNT/SHH Axonal Guidance Signaling Pathway	1.51E-07	2.041	24
7	Neuropathic Pain Signaling in Dorsal Horn Neurons	1.82E-07	2.065	19
8	Glutaminergic Receptor Signaling Pathway (Enhanced)	2.57E-07	0.493	38
9	Ephrin Receptor Signaling	9.12E-07	0.471	27
10	Orexin Signaling Pathway	9.55E-07	1.826	30

GR-A Unique 636
(Dependence: GR-A when GR NTD isoforms deficient)

Rank	Nervous System Signaling Pathways	p-value	z-score	Genes
1	Regulation of Actin-based Motility by Rho	4.17E-05	0	11
2	Semaphorin Neuronal Repulsive Signaling Pathway	4.90E-04	0.333	11
3	Ephrin Receptor Signaling	6.61E-04	0.333	13
4	Myelination Signaling Pathway	3.16E-03	-1	16
5	Neuroinflammation Signaling Pathway	3.31E-03	-1.732	16
6	Reelin Signaling in Neurons	4.17E-03	-1	9
7	Axonal Guidance Signaling	6.46E-03	n/a	21
8	Semaphorin Signaling in Neurons	1.00E-02	n/a	5
9	Synaptogenesis Signaling Pathway	1.20E-02	-0.535	14
10	Neuregulin Signaling	1.70E-02	n/a	7

B

WT Unique 465
(Dependence: GR NTD isoforms)

Rank	Biological Functions	p-value	Genes
1	Organismal Survival	3.3E-12-4.73E-05	145
2	Cardiovascular System Development and Function	7.94E-12-4.98E-04	109
3	Organismal Development	7.94E-12-6.14E-04	212
4	Tissue Development	1.48E-11-6.14E-04	195
5	Embryonic Development	9.51E-11-5.87E-04	167
6	Nervous System Development and Function	1.33E-09-6.14E-04	133
7	Tissue Morphology	9.97E-09-5.75E-04	125
8	Endocrine System Development and Function	3.63E-08-3.33E-04	60
9	Hematological System Development and Function	6.65E-08-6.07E-04	117
10	Lymphoid Tissue Structure and Development	6.65E-08-5.87E-04	74

WT/GR-A Common 1305
(Dependence: GR-A)

Rank	Biological Functions	p-value	Genes
1	Organismal Survival	2.62E-45-3.84E-11	486
2	Tissue Morphology	4.22E-38-2.66E-09	396
3	Cardiovascular System Development and Function	5.76E-36-2.5E-09	270
4	Organismal Development	5.76E-36-2.5E-09	647
5	Nervous System Development and Function	5.52E-35-2.43E-09	388
6	Tissue Development	5.52E-35-3.06E-09	601
7	Embryonic Development	1.12E-33-2.43E-09	475
8	Hematological System Development and Function	3.59E-31-2.03E-09	355
9	Connective Tissue Development and Function	4.05E-28-3.06E-09	319
10	Immune Cell Trafficking	9.56E-27-1.58E-09	187

GR-A Unique 636
(Dependence: GR-A when GR NTD isoforms deficient)

Rank	Biological Functions	p-value	Genes
1	Lymphoid Tissue Structure and Development	5.99E-26-1.81E-05	152
2	Tissue Morphology	5.99E-26-1.72E-05	187
3	Hematological System Development and Function	9.84E-25-1.81E-05	212
4	Cardiovascular System Development and Function	4.78E-23-1.34E-05	127
5	Organismal Development	4.78E-23-1.49E-05	265
6	Organismal Survival	3.38E-21-8.57E-06	232
7	Immune Cell Trafficking	1.02E-20-1.81E-05	138
8	Hematopoiesis	8.96E-20-1.55E-05	118
9	Tissue Development	8.96E-20-1.72E-05	249
10	Embryonic Development	3.51E-18-1.03E-05	184

FIGURE 4 | Legend on next page.

FIGURE 4 | Signaling pathways and biological functions associated with the 3 groups of Dex-regulated genes in the hippocampus of WT and GR-A knockin mice. (A) Top nervous system signaling pathways most significantly associated with the 465 genes uniquely regulated by Dex in the WT hippocampus, the 1305 genes commonly regulated by Dex in both the WT and GR-A knockin hippocampus, and the 636 genes uniquely regulated by Dex in the GR-A knockin hippocampus as determined by IPA. The *z*-score predicts the overall effect the gene changes have on the signaling pathway. Positive *z*-scores are indicative of activation of the pathway and negative *z*-scores are indicative of inhibition of the pathway. *Z*-scores greater than the absolute value of 2 are considered significant. Pathways for which no activity prediction can be made are indicated with an "n/a" for not applicable. (B) Top biological functions most significantly associated with the 465 genes uniquely regulated by Dex in the WT hippocampus, the 1305 genes commonly regulated by Dex in both the WT and GR-A knockin hippocampus, and the 636 genes uniquely regulated by Dex in the GR-A knockin hippocampus as determined by IPA. The range in *p*-values reflects the *p*-values of the multiple lower-level functions that comprise the indicated high-level functional category.

reporter assay which directly measures protein–protein interactions in living cells. HEK-293 cells were transfected with various combinations of plasmids expressing GR isoform-LgBit or GR isoform-SmBit fusion proteins (Figure 7A). The interaction of these proteins was monitored before and after the addition of Dex for a total of 65 min. We first examined the ability of the GR-A isoform to homodimerize and heterodimerize with the GR NTD isoforms (Figure 7B). In cells co-expressing the two GR-A fusion proteins, Dex treatment resulted in a robust increase in luminescence indicative of GR-A homodimerization. The enhanced signal began approximately 5 min after addition of Dex and persisted throughout the remaining incubation. In cells co-expressing GR-A and GR-B, a similar Dex-dependent enhancement of the luciferase signal was observed indicating that GR-A can heterodimerize with the GR-B isoform. Dex treatment of cells co-expressing GR-A and GR-C3 or GR-A and GR-D3 led to a small increase in luciferase signal suggesting GR-A can also heterodimerize with GR-C3 and GR-D3, but these interactions are weak compared to its heterodimeric interaction with GR-B.

We next evaluated the ability of the GR NTD isoforms to interact with one another (Figure 7C). In cells co-expressing the two GR-B fusion proteins, Dex treatment resulted in an enhanced luciferase signal similar to that measured for the GR-A homodimers, suggesting the GR-B isoforms associate as a homodimer. A significant Dex-dependent interaction was also observed between GR-B and GR-C3 and between GR-B and GR-D3 suggesting GR-B, like GR-A, can heterodimerize with these other GR NTD isoforms but the interaction is comparatively weak. In cells co-expressing the two GR-C3 fusion proteins or the two GR-D3 fusion proteins, no significant luciferase signal was observed with Dex treatment suggesting these GR NTD isoforms do not form stable homodimeric complexes. Similarly, we detected no evidence for stable heterodimeric complexes between GR-C3 and GR-D3. These data demonstrate that the GR NTD translational isoforms exhibit marked differences in their ability to interact with GR-A and with each other, providing a mechanistic basis by which these receptor isoforms can uniquely impact the glucocorticoid signaling profile and transcriptome.

4 | Discussion

The single GR gene gives rise to multiple NTD translational isoforms that are highly conserved across species [6, 21]; however, the role these isoforms play in mediating the actions of glucocorticoids in vivo remains poorly understood. To elucidate the physiological function of the GR NTD translational isoforms, we developed mice that express the classic full-length GR-A isoform but lack the 7 NTD translational subtypes. Analysis of the hippocampal transcriptome in WT and GR-A knockin mice treated acutely with Dex revealed three sets of glucocorticoid-regulated genes: genes dependent on the GR NTD translation isoforms, genes dependent only on GR-A, and genes dependent on GR-A under conditions of GR NTD translational isoform deficiency. All three gene sets were strongly associated with axonal guidance signaling and nervous system development. Provocatively, the genes requiring the GR NTD translational isoforms for their regulation by Dex were preferentially associated with synaptic function, circadian rhythm, and cognition. Consistent with these gene enrichment results, the GR-A knockin mice showed enhanced corticosterone levels in response to stress and increased fear-motivated contextual learning. The GR NTD translational isoforms differed in their capacity to form homodimeric complexes and heterodimeric complexes with GR-A and each other, providing a mechanistic basis for their unique glucocorticoid signaling profiles. These data demonstrate that the GR NTD translational isoforms contribute to the hippocampal actions of glucocorticoids by creating additional heterogeneity in glucocorticoid signaling profiles.

Our finding that stress-induced corticosterone levels are elevated in the GR-A knockin mice suggests that the GR NTD translational isoforms play an important role in the glucocorticoid negative feedback response. Glucocorticoids terminate stress-induced rises in HPA activity by negative feedback mechanisms occurring at multiple sites including the hippocampus, paraventricular nucleus (PVN), and anterior pituitary [22–25]. The actions of glucocorticoids on the hippocampus lead to reduced activity of PVN neurons and decreased secretion of corticotropin-releasing hormone (CRH). The reduced levels of CRH lead to reduced secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary and decreased production of glucocorticoids from the adrenal gland. The GR NTD translational isoforms preferentially target genes important for circadian rhythm signaling in the hippocampus. Given the close connection and extensive overlap of the circadian clock and stress response systems [26], many of the circadian rhythm genes targeted by the GR NTD isoforms in the hippocampus may also function to modulate HPA axis activity in times of stress by contributing to the glucocorticoid-dependent suppression of PVN neuronal activity. A deficiency in these gene changes in the GR-A knockin mice would impair the negative feedback response and result in elevated stress-induced levels of corticosterone. It will be important for future studies to investigate whether these genes are dysregulated in intact GR-A knockin mice under physiological conditions when glucocorticoids are low (circadian nadir) and elevated (circadian peak and/or stress). Elucidating the factors and mechanisms involved in glucocorticoid negative feedback is a critical goal of current research because deficits in this response are thought to underlie a variety of stress-related psychological disorders including

A

WT Unique 465
(Dependence: GR NTD isoforms)

Rank	Nervous System Annotations	p-value	Genes
1	Synaptic transmission	5.79E-08	28
2	Neurotransmission	1.95E-07	31
3	Morphology of dendritic spines	1.61E-06	9
4	Morphology of dendrites	2.36E-06	12
5	Synaptic transmission of brain cells	3.28E-06	9
6	Formation of dendrites	5.59E-06	20
7	Dendritic growth/branching	1.20E-05	22
8	Synaptic transmission of cerebral cortex cells	1.85E-05	8
9	Length of dendritic spines	4.30E-05	5
10	Extension of axons	7.37E-05	10
11	Long term synaptic depression	1.85E-04	10

WT/GR-A Common 1305
(Dependence: GR-A)

Ranks	Nervous System Annotations	p-value	Genes
1	Formation of dendrites	6.72E-10	48
2	Neurotransmission	7.15E-10	69

GR-A Unique 636
(Dependence: GR-A when GR NTD isoforms deficient)

No significant Nervous System Annotations

B

WT Unique 465
(Dependence: GR NTD isoforms)

Rank	Behavioral Annotations	p-value	Genes
1	Anxiety	1.74E-07	25
2	Cognition	7.07E-07	34
3	Circadian rhythm	1.18E-06	13
4	Learning	1.60E-06	31
5	Memory	6.17E-06	22
6	Spatial learning	4.65E-05	15
7	Locomotion	1.03E-04	22
8	Feeding	4.11E-04	19
9	Social behavior	4.98E-04	10

WT/GR-A Common 1305
(Dependence: GR-A)

Rank	Behavioral Annotations	p-value	Genes
1	Anxiety	2.48E-11	57

GR-A Unique 636
(Dependence: GR-A when GR NTD isoforms deficient)

No significant Behavioral Annotations

FIGURE 5 | Legend on next page.

FIGURE 5 | Specific nervous system and behavioral annotations associated with the 3 groups of Dex-regulated genes in the hippocampus of WT and GR-A knockin mice. (A) Nervous system development and function annotations related to synapse most significantly associated with the 465 genes uniquely regulated by Dex in the WT hippocampus, the 1305 genes commonly regulated by Dex in both the WT and GR-A knockin hippocampus, and the 636 genes uniquely regulated by Dex in the GR-A knockin hippocampus as determined by IPA. No annotations related to synapse were significantly associated with the 636 genes uniquely regulated by Dex in the GR-A knockin hippocampus. (B) Behavioral annotations most significantly associated with the 465 genes uniquely regulated by Dex in the WT hippocampus and the 1305 genes commonly regulated by Dex in both the WT and GR-A knockin hippocampus as determined by IPA. Behavior was not significantly associated with the 636 genes uniquely regulated by Dex in the GR-A knockin hippocampus.

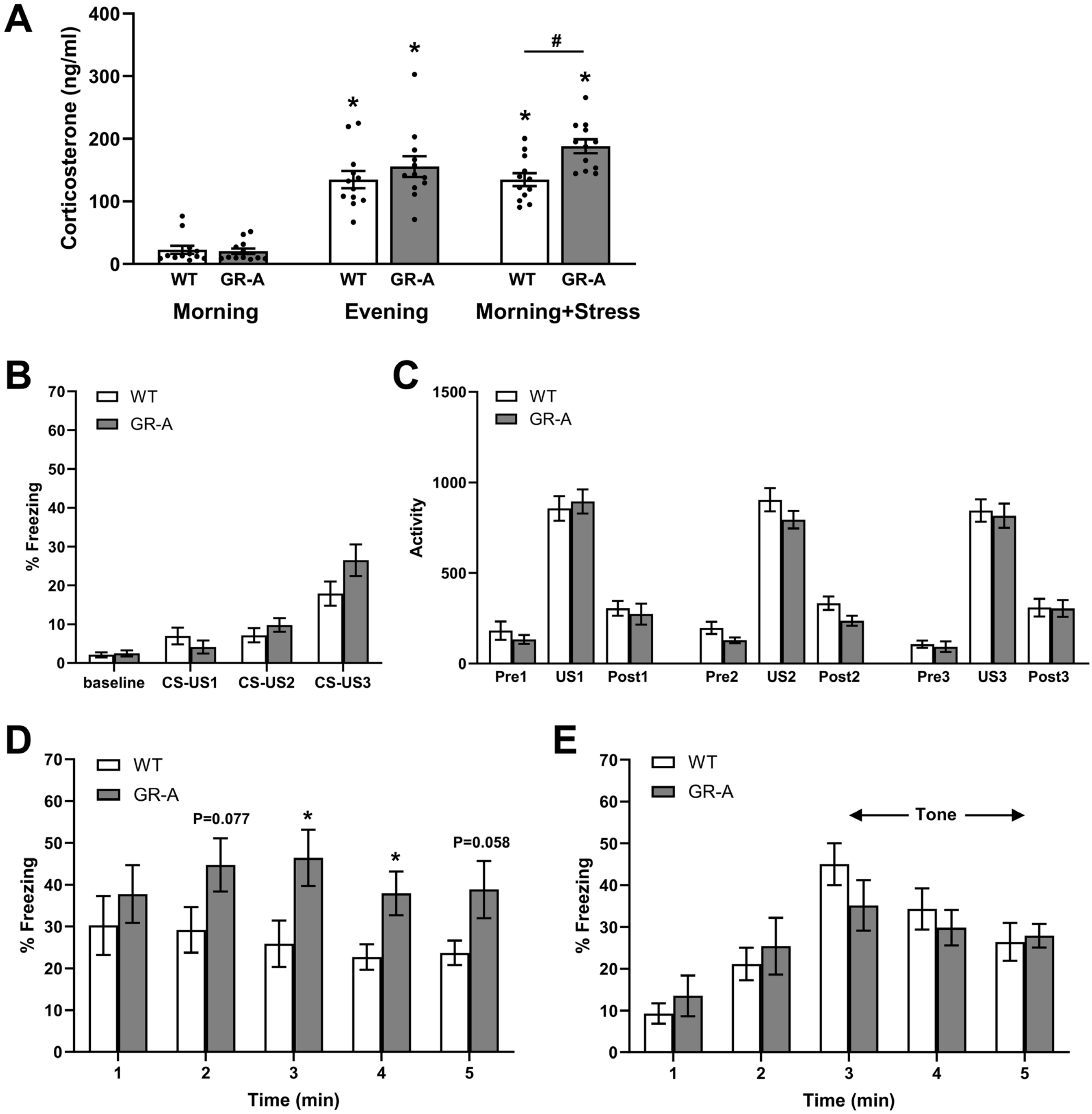

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FIGURE 6 | GR-A knockin mice exhibit alterations in HPA axis activity and fear-motivated contextual learning. (A) Corticosterone levels at the circadian nadir (morning), peak (evening), and in the morning immediately following 30 min of restraint stress (morning + stress) in WT and GR-A knockin mice. Data are mean ± SEM (n = 12 mice per genotype). A two-way repeated measures ANOVA followed by Sidak's (within genotype) or Tukey's (across genotypes) multiple comparisons post hoc test was performed to determine significance [time × genotype interaction, $F(2,44) = 3.833$, $p = 0.0292$; main effect of time, $F(1.671,36.76) = 114.7$, $p < 0.0001$; main effect of genotype, $F(1,22) = 4.923$, $p = 0.0371$]. $^*p < 0.05$ for evening compared to morning of same genotype and for morning + stress compared to morning of same genotype. $^{\#}p < 0.05$ for GR-A compared to WT. (B) Levels of freezing for the WT and GR-A knockin mice during the first 2 min (baseline) and during each of 3 tone-shock presentations (CS-US) of the fear conditioning training session on Day 1. Data are mean ± SEM (n = 13–14 mice per genotype). A two-way repeated measures ANOVA was performed to determine significance [time × genotype interaction, $F(3,75) = 3.248$, $p = 0.0265$]. Fisher's PLSD post hoc analysis at each time period revealed no significant differences between genotypes. (C) Levels of activity of the WT and GR-A knockin mice during the 2-s foot shock (US), as well as in the preceding (Pre) and following (Post) 2-s periods, for each of the 3 tone-shock presentations during the training session on Day 1. The unit of activity is based on number of pixel changes in the video record during the 2-s intervals, with counts taken by the automated image tracking system. Data are mean ± SEM (n = 13–14 mice per genotype). A two-way repeated measures ANOVA revealed no significant differences between genotypes. (D) Context-dependent learning was evaluated using the conditioned fear test. After training on Day 1, mice were placed back in the original conditioning chamber and levels of freezing were determined across a 5-min session on Day 2. Data are mean ± SEM (n = 13–14 mice per genotype). A two-way repeated measures ANOVA followed by Fisher's PLSD post hoc analysis was performed to determine significance [main effect of genotype, $F(1,25) = 5.292$, $p = 0.0301$]. $^*p < 0.05$ for GR-A compared to WT. (E) Cue-dependent learning was evaluated using the conditioned fear test. After training on Day 1, mice were placed in modified conditioning chamber and levels of freezing were determined before and during the acoustic stimulus for the 5-min session on Day 3. Data are mean ± SEM (n = 13–14 mice per genotype). A two-way repeated measures ANOVA revealed no significant differences between genotypes.

schizophrenia, bipolar disorder, generalized anxiety, and major depressive disorder [25, 27].

The GR-A knockin mice exhibited increased contextual learning in a fear conditioning test. Fear memories are crucial for adaptation to stress, and many studies have shown that glucocorticoids regulate multiple stages of the fear memory process [28, 29]. One of the main brain areas targeted by glucocorticoids for these effects on emotional memories is the hippocampus. In fear conditioning experiments, increased levels of glucocorticoids have been shown to enhance the formation of contextual memories whereas reduced levels of glucocorticoids or pharmacological blockade of GR impair their consolidation [28, 30–32]. Our discovery of increased fear learning in the GR-A knockin mice suggests that the GR NTD translational isoforms normally function to limit the formation of contextual fear memories. Consistent with this effect, the GR NTD isoforms preferentially targeted genes associated with LTD and cognition. LTD is the persistent weakening of a synapse that functions to limit the expression of long-term potentiation (LTP), which is the process of synaptic strengthening that underlies learning and memory [33, 34]. LTD is also known to be enhanced by glucocorticoids [35]. An enhancement of LTD would limit fear memories and serve as a safeguard to prevent emotional memories from becoming overly consolidated since intensified expressions of fear can be debilitating. Heightened fear expressions toward stimuli previously associated with trauma are a common aspect of post-traumatic stress disorder (PTSD) [36]. Dysregulated fear memory processing may also play a significant role in other psychiatric conditions, such as phobias, panic disorder, obsessive compulsive disorder, and generalized anxiety disorder [37]. Findings from the GR-A knockin mice raise the intriguing possibility that deficiencies in the expression and/or activity of the GR NTD translational isoforms may contribute to the etiology of disorders characterized by inappropriate fear responses.

Our transcriptomic studies on the GR-A knockin mice show that the GR NTD translational isoforms are required for a large cohort of genes to be regulated by glucocorticoids. This regulation could be achieved directly by the individual actions of one or more of the GR NTD translational isoforms. Indeed, in vitro studies have shown that each of the GR translational isoforms can regulate distinct gene sets [6, 8, 9]. In addition, however, the regulation may involve cooperative actions of the GR NTD translational isoforms with each other and/or with GR-A. To better understand at a mechanistic level how the GR NTD translational isoforms modify the transcriptional response of target genes, we evaluated the ability of these isoforms to form homodimeric complexes and heterodimeric complexes with each other and with GR-A. We discovered that GR-A and GR-B can readily associate as homodimers and heterodimers in response to Dex treatment. In contrast, GR-A and GR-B exhibited much weaker heterodimeric interactions with GR-C3 and GR-D3. Surprisingly, the GR-C3 and GR-D3 isoforms showed no significant capacity to homodimerize nor heterodimerize with each other. The GR residues important for dimerization have been reported to reside in the DBD and LBD that are both intact in all the GR NTD translational isoforms [38]. The compromised ability of the GR-C3 and GR-D3 isoforms to form homodimeric and heterodimeric complexes in response to Dex suggests that residues in the GR NTD may also play an important role in this process. Our findings indicate that GR-A and GR-B have the full repertoire of receptor configurations (monomer, homodimer, and heterodimer) to engage target genes and regulate their transcription whereas GR-C3 and GR-D3 appear to predominantly function as monomers. The distinct abilities of GR-A and the GR NTD translational isoforms to function as monomers, homodimers, and heterodimers suggests each receptor species has its own unique genetic signature which greatly expands the heterogeneity in glucocorticoid-dependent transcriptional responses.

Analysis of the Dex-regulated genes in the hippocampus of the WT and GR-A knockin mice suggests that the majority of the genomic actions of glucocorticoids are accomplished by GR-A acting independently of the GR NTD translational isoforms. A total of 1305 genes, representing approximately 70% of the

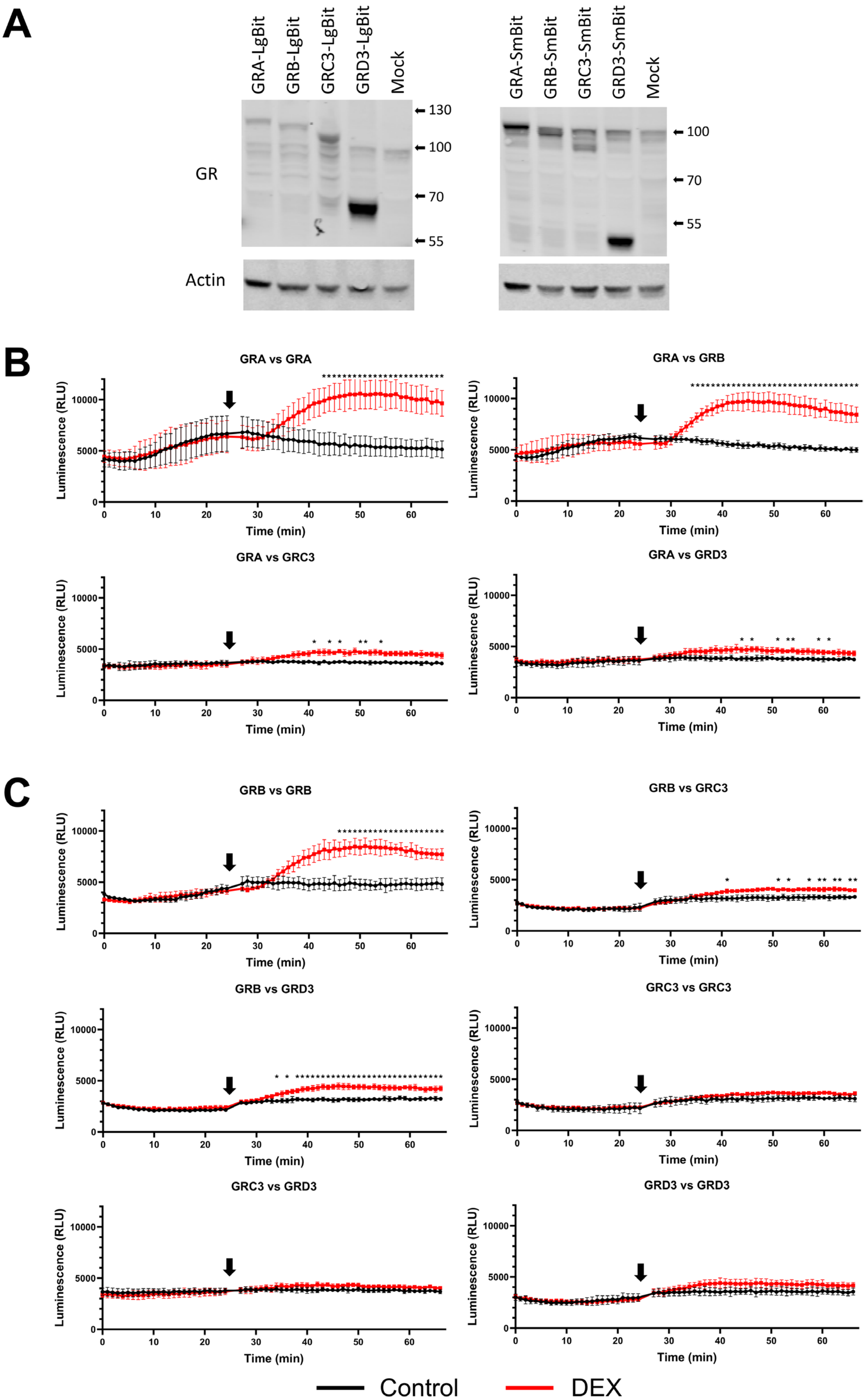

FIGURE 7 | Legend on next page.

FIGURE 7 | GR NTD translational isoforms have unique capacities to homodimerize and heterodimerize. (A) Protein expression levels of GR isoform fusions with the Nanobit tags (LgBit or SmBit) were compared by immunoblot analysis of HEK293 cell extracts using anti-GR and anti-actin (loading control) antibodies. (B, C) GR-A vs. GR-A, GR-A vs. GR-B, GR-A vs. GR-C3, and GR-A vs. GR-D3 isoform interactions (B) and GR-B vs. GR-B, GR-B vs. GR-C3, GR-B vs. GR-D3, GR-C3 vs. GR-C3, GR-C3 vs. GR-D3, and GR-D3 vs. GR-D3 isoform interactions (C) were evaluated in living HEK293 cells by reconstitution of Nanoluc luciferase activity. Nanoluc luciferase substrate was added to cells at 0 min and luminescence was measured each minute for 65 min. At 25 min, Dex (final concentration 100 nM) was added to cells as indicated by the arrow. The red lines represent luminescence from Dex-treated cells while the black lines represent luminescence from control cells. Data are mean ± SD ($n = 3–5$). A two-way repeated measures ANOVA followed by Sidak's multiple comparisons post hoc test was performed to determine significance. *$p < 0.05$ for Dex cells compared to control cells. The two-way repeated measures ANOVA statistical results for the factors time, treatment, and their interaction are shown for each graph in Figure S7.

glucocorticoid transcriptome, were commonly regulated by Dex in both the WT and GR-A hippocampus. These genes appear to depend only on GR-A for their regulation by Dex since the gene regulatory profile is similar whether the GR NTD translational isoforms are present or not. These genes were preferentially associated with CREB and glutaminergic signaling. CREB signaling affects many processes in the adult hippocampus such as neurogenesis, synaptic plasticity, and neuron survival, and glutamine is the chief excitatory neurotransmitter in the hippocampus. Unexpectedly, our global transcriptomic analysis also revealed that GR-A gained the ability to regulate a large cohort of genes in the absence of the GR NTD translational isoforms. These genes were preferentially associated with neuroinflammation. This finding suggests that the GR NTD isoforms normally function to limit the activity of GR-A on a subset of genes important for inflammatory responses in the brain. The GR NTD translational isoforms may accomplish this inhibition of GR-A on specific target genes by occupying glucocorticoid responsive elements (GREs) and precluding GR-A from binding these sites and/or by forming a heterodimer with GR-A that is deficient in its ability to bind DNA or altered in its recruitment of transcriptional coregulators. It is also possible that the methionine to isoleucine substitutions necessary to make the GR-A knockin mice may have altered the transcriptional activity of GR-A on certain genes. This scenario appears unlikely, however, since GR-A with the seven methionine to isoleucine substitutions has been shown in cells to be similar to wild-type GR in expression level, half-life, subcellular distribution, activation of reporter genes, capacity to bind GREs and recruit coregulators to various endogenous genes, and functional ability to induce apoptosis [6, 7].

The ability of the GR NTD translational isoforms to modulate the glucocorticoid transcriptome in the hippocampus makes it important to understand the factors that control their expression and/or activity. Many studies have demonstrated that the expressed complement of GR NTD isoforms in specific cells and tissues can change under certain conditions. The selective estrogen-related receptor beta/gamma agonist promotes a preferential increase in the GR-D isoforms in differentiated murine skeletal muscle cells [39], and mitogen activation of human primary T cells results in a selective upregulation of the GR-C isoforms [9]. Alterations in the expression of the GR NTD isoforms have also been observed in the human placenta due to gestational age, fetal sex, and maternal alcohol consumption [10, 11], and in the human brain during development and during the aging process [14]. Moreover, the GR-D isoforms were found to exhibit a selective increase in expression in specific regions of the brain in patients with schizophrenia and bipolar disorder [12, 13]. Polymorphisms in the GR gene and heterogeneity in the 5′untranslated region of the GR mRNA can influence the efficiency with which particular GR start codons are utilized for translation and impact the GR NTD isoform expression pattern [40–42]. The phosphorylation status of GR-A is known to impact its transcriptional activity and its half-life, and the GR NTD translational isoforms exhibit patterns of phosphorylation distinct from GR-A [8]. This suggests that the activity and expression levels of the GR subtypes in a given cell may also be finely tuned by the activity of specific kinases and phosphatases. A limitation of our study is that it examined the glucocorticoid transcriptome only in the hippocampus of male mice. Therefore, it will be important for future studies to assess the glucocorticoid signaling profile of the GR-A knockin mice in other tissues and in female mice to gain a more complete understanding of how the GR NTD isoforms modulate glucocorticoid responses.

In summary, we have developed a novel mouse model that expresses the classic GR-A isoform but not the GR NTD translational isoforms. These mice reveal a critical role for the GR NTD translational isoforms in the glucocorticoid-dependent regulation of hippocampal genes important for HPA axis activity and fear-motivated contextual learning. Moreover, they show that the GR NTD translational isoforms are essential for generating heterogeneity in glucocorticoid signaling. These findings suggest that modulating the expression and/or activity of the GR NTD translational isoforms may provide a new strategy for treating disease.

Author Contributions

R.H.O.: conceptualization, methodology, investigation, formal analysis, writing – original draft, writing – review and editing. T.S.: methodology, investigation, formal analysis, writing – original draft. S.R.: methodology, investigation. V.D.N.: methodology, investigation, formal analysis. D.R.G.: methodology. J.L.: formal analysis. M.K.R.: methodology, funding acquisition. S.S.M.: methodology, investigation, formal analysis, writing – original draft, writing – review and editing, funding acquisition. J.A.C.: conceptualization, methodology, writing – review and editing, funding acquisition.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The RNA-seq data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with the accession number GSE298763.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** GR-D translational isoforms are expressed in WT but not GR-A knockin mice. **Figure S2:** Volcano plots of Dex-regulated genes in the hippocampus of WT and GR-A knockin mice. **Figure S3:** Biological functions associated with the 3 groups of Dex-regulated genes in the hippocampus of WT and GR-A knockin mice. **Figure S4:** GR-A knockin mice exhibit no alterations in anxiety-like behavior. **Figure S5:** Evaluation of GR-A knockin mice for motor coordination and learning using the acclerating rotarod test. **Figure S6:** Evaluation of GR-A knockin mice for auditory function, reactivity to environmental stimuli, and sensorimotor gating using the acoustic startle test. **Figure S7:** The two-way repeated measures ANOVA statistical results for the factors time, treatment, and their interaction for Nanobit interaction graphs presented in Figure 7. **Table S1:** The WT/GR-A common 1305 genes that depend on the GR-A isoform for their regulation by Dex. **Table S2:** The WT unique 465 genes that depend on the GR NTD isoforms for their regulation by Dex. **Table S3:** The GR-A unique 636 genes that depend on the GR-A isoform under conditions of GR NTD isoform deficiency for their regulation by Dex. **Table S4:** Dex-regulated genes in IPA signaling pathways preferentially associated with the WT unique 465, WT/GR-A common 1305, and GR-A unique 636 gene sets.