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Regulatory effects of Maternal Immune Activation and Environmental Enrichment on Glucocorticoid Receptor and FKBP5 expression in stress-sensitive regions of the offspring brain

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Abstract

A mother's exposure to immune challenge during pregnancy is well known to be a detrimental factor to the development of the offspring's brain and an impetus for neuropsychiatric disorders. Previous studies have shown that these adverse events can dysregulate the stress response machinery. Two crucial components of the stress axis considered to be affected have been targets in these studies: the glucocorticoid receptor (GR), and FKBP5 which regulates GR activity. The implementation of interventions such as Environmental Enrichment (EE) have shown positive results in protecting the brain against the consequences associated with gestational insults. In light of this, we investigated the transcriptional regulation of GR and FKBP5 from 6 stress-sensitive brain regions of the offspring using a rat model of maternal immune activation (MIA). Furthermore, we analyzed the effect of an enriched environment on their expression. We found an increase in FKBP5 in MIA rats in 5 brain regions. RT-qPCR analysis of MIA's effect on GR yielded insignificant results. However, we found that EE increased GR expression in the medial preoptic area which could be indicative of a positive regulation by EE. This study provides evidence of the impact of both gestational insult and EE on the regulation of stress responsive genes in the developing brain.

Keywords

Early life stress; HPA axis; Developing brain; Psychotic Disorders

Introduction:

Models of prenatal immune activation have been providing a clear association between maternal infection during pregnancy with adverse brain development and neurobiological disruptions, resulting in early life vulnerability to psychiatric disorders for the offspring

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(Meyer et al., 2009; Gumusoglu & Stevens, 2018; Sørensen et al., 2008). A better understanding of the molecular mechanisms caused by maternal immune activation (MIA) in the brains of offspring would lead to the identification of the key pathological players responsible for the etiology of psychiatric illnesses as well as the development of more targeted clinical treatments. This study attempts to identify both.

During stressful conditions, our body naturally triggers a neuroendocrinal response through the hypothalamic-pituitary-adrenal (HPA) axis, our central stress response system, to adapt and restore homeostasis (allostasis). The paraventricular nucleus of the hypothalamus secretes corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) which in turn stimulate the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH is released into the circulation and travels down to the adrenal glands where it stimulates the synthesis and secretion of glucocorticoids (GCs) from the zona fasciculate of the adrenal cortex along with other adrenal steroids, namely DHEA and mineralocorticoids from the zona glomerulosa and reticularis, respectively. The released GCs in the systemic circulation will then affect a constellation of physiological processes, namely metabolism (Vegiopoulos & Herzig, 2007), water and electrolyte balance (Hawkins et al., 2012), the immune response (Cruz-Topete & Cidlowski, 2014; Bosscher & Haegeman, 2008), growth (Donatti et al., 2011), cardiovascular function (Cruz-Topete et al., 2016; Nussinovitchet al., 2010), mood and cognitive functions (Farrell & O'Keane, 2016; Joëls., 2011; Tatomir et al., 2014), reproduction (Whirledge & Cidlowski, 2017), and development Fowden & Forehead,2015). Glucocorticoids in response negatively regulate the HPA-axis. Under basal, unstressed conditions, the dynamics of the HPA axis is characterized by both an ultradian and a circadian rhythm of hormone secretion with a peak in levels of glucocorticoids early in the morning for diurnal species (Spiga et al., 2014; Dickmeis et al., 2013).

The effects of glucocorticoids in the brain are primarily mediated by the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), both members of the nuclear receptor (NR) superfamily acting as transcription factors (McEwen et al., 1986; De Kloet., 1991). In the late 1980s, Chao and colleagues found that GR and MR are particularly abundant in stress-sensitive regions of the brain, namely the prefrontal cortex and hippocampus (Chao et al., 1989; Saánchez et al., 2000). It appears that MR shows high affinity for glucocorticoids as well as aldosterone. In mammals and rodents, MR has 5- to 10-fold higher affinity for corticosterone than does GR (Breuner and Orchinik., 2009; De Kloet et al., 1975; McEwen et al., 1968). Given the pathological implications of models of maternal immune activation in neurodevelopment, the lower affinity that GR has for GCs does not constitute an important factorial consideration in this study. The particular interest in GR lies in the influence of GCs on immune function, which is primarily mediated by the glucocorticoid receptor (Marchetti et al., 2001). Also, GCs differential selective affinity for MR and GR suggests that GR may be the receptor that mostly mediates the stress response when GCs are abundant whereas MR mediates basal conditions. The activation of the immune system in a MIA model, following administration of lipopolysaccharide (LPS) is accompanied by a pronounced and long-lasting elevation in the circulating levels of GCs. In his review, Barnes suggested that the inflammatory genes that are activated in chronic inflammatory diseases are suppressed through the binding of the liganded GR to coactivator molecules and recruitment of histone deacetylase-2 (HDAC2) to the activated transcription

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complex (Barnes., 2010), one of a number of epigenetic effects of GR activity. Paradoxical findings to this immunosuppressive mechanism of GR in chronic inflammatory diseases, revealed that the anti-inflammatory properties of GR are weakened while pro-inflammatory responses are enhanced in caregivers of brain cancer patients (Rohleder et al., 2009; Miller et al., 2008). Caregiving has recently been characterized as a model of chronic stress (Pinquart and Sorensen, 2003) and this evidence challenges our conventional understanding of the effects of GR on immune functions during chronic stress.

The co-chaperone of hsp90, FK506-binding protein 51 or FKBP5 for short, is an important element in this study considering its effect on GR function. FKBP5 regulates GR sensitivity by acting as a negative-feedback regulator of the receptor (GR). Increased expression of FKBP5 reduces available GRs and promote glucocorticoid resistance. Studies on psychological stress have shown that specific FKBP5 single-nucleotide polymorphic variants such as rs1360780 and rs9296158 affect susceptibility to PTSD after early-life trauma through modifying GR binding to this gene (Binder EB et al., 2008; Mehta D et al., 2011).

Environmental enrichment (EE) is an enriched therapeutic housing filled with novel toys and an increased opportunity for social, sensory, and cognitive stimulations. Accumulating studies showed that life-long exposure to environmental enrichment (EE), a translational intervention (Woo & Leon, 2013; Woo et al., 2015; Aronoff et al., 2016; Downs et al., 2018; Morgan et al., 2013; Morgan et al., 2015; Purpura et al., 2014), starting prior to breeding and extending through gestation until the postpartum period protects offspring against some effects of MIA (Connors et al., 2014). More recent evidence in rodents showed that EE specifically protects placental and fetal brain functioning following MIA-associated glucocorticoid programming (Nunez Estevez et al., 2020). In addition, the rehabilitating and supportive effects of a life-long EE exposure have been found to attenuate the MIA-induced downregulation of hippocampal GR in juvenile male rats (Connors et al., 2014). Thus, a supportive environment may promote resilience against the GC mediated effects of insults like MIA.

The aims of this present study were to investigate the effect of early life stress, in the form of maternal infection during gestation, on the expression of the glucocorticoid receptor and FKBP5 as well as the direct effect that environmental enrichment had on the MIA-associated expression of the glucocorticoid receptor and FKBP5 in stress-sensitive regions of the offspring brain following treatment with the bacterial toxin, lipopolysaccharide. Based on the overwhelming volume of research revolving around the underlying mechanisms of psychiatric disorders following early life adversities, it is more than necessary to scrutinize the interplay between the stress hormone receptor, GR and factors promoting resiliency, such as a suitable environment.

Materials & Method:

Animal Care

Male and female Sprague-Dawley rats (N=42, males=12, females=30) were obtained from Charles River Laboratories, Wilmington, MA and maintained at 20 °C under a 12:12h light/dark cycle with ad libitum food and water at MCPHS University for the remaining

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of the rearing protocol. Female rats were then randomized in pairs and exposed to either Environmental Enrichment (EE; large multi-level cage with toys changed 2 times weekly, tubes, chew bone, Nestlets and ramps; Critter Nation, Muncie IN) or Animal Care Control (ACC; standard cage with tube, chew bone, and Nestlets) housing treatment. After one week, one male rat was introduced to each cage with females and allowed to breed. Vaginal samples were collected to confirm pregnancy. On gestational day 11 (G11), each ACC and EE dams received an i.p. injection of either LPS (Escherichia coli, serotype 026:B6; L-3755, Sigma, St. Louis, MO; 100 lg/kg) in pyrogen-free saline, or an equivalent volume of pyrogen-free saline. On postnatal day 22 (P22), one female and one male pup from each dam were sacrificed and their brains removed and stored in dry ice. The details of MIA with LPS and EE conditioning are described by Connors et al. (2014) and in the Maternal Immune Activation Model Reporting Guidelines Checklist (Supplementary Table 1). Animal procedures were approved by the MCPHS University Institutional Animal Care and Use Committee and carried out in accordance with the recommendations outlined by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Brain Region Extraction

The brains were stored at −80°C until use. The frozen brains were then mounted and sectioned on a Leica cryostat. The dorsal striatum (DST), dorsal hippocampus (DHipp), ventral hippocampus (VHipp), medial pre-optic area (MPOA), nucleus accumbens (NAcc), and prefrontal cortex (PFC) regions were extracted from the brains using a Harris Uni-Core 2.00mm brain punch tool. 200um brain punches from all six regions were taken for all animals. The extracted brain tissues were stored in separate 1.5mL Eppendorf tubes at −80°C until molecular analysis.

RT qPCR

RNA from all brain regions was extracted using a Zymo Research Quick-RNA miniprep kits and quantified on a NanoDrop. The measured concentrations were recorded. cDNA was synthesized using a reverse transcriptase kit (Applied Biosystems High-Capacity cDNA Reverse Transcription) following manufacturer's instructions. The cDNA obtained was quantified on a NanoDrop and normalized to a concentration of 1ug/ul and stored at 4°C for PCR testing. The remaining samples were stored back at −20°C. The 96-well PCR plates were prepared following a layout provided by the Hunter Lab. The reaction volume per well was 15ul. Each well contained 5.5ul of cDNA or 5.5ul of nuclease free water (control wells), 7.5ul of Thermo Fisher PowerUp SYBR Green Master Mix, and 1ul of primers for *GR* and *FKBP5* purchased from Integrated DNA Technologies or 1ul of housekeeping gene GAPDH (control). Each qPCR reaction was run in duplicates using the Applied Biosystems StepOnePlus System. Please refer to Table 1 for primer sequences.

Statistical Analyses

Statistical analyses were performed using the software package Statistical Software for the Social Science (SPSS) version 27.0 (IBM, Armonk, NY). Before performing any statistical analysis, data points that fell above or below 2 standard deviations were excluded. Two-way (gestational treatment/housing) and three-way (gestational treatment/housing/sex) ANOVAs were conducted with post hoc tests using pairwise t tests and Levene's test for homogeneity

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of variance to examine main and interaction effects on *FKBP5* and *GR* expression. The $2^{\Delta\Delta Ct}$ method was used to calculate the logarithmic fold change of the target gene expression in the target (LPS) and reference (saline) samples relative to the saline-treated animals, normalized to GAPDH (see Supplementary Figure 2 for brain GAPDH expression across conditions). Male and female data were collapsed together unless there was a significant effect of sex on the measures. The experimental data was represented as box and whisker plots with superimposed individual data points. Alpha levels were set at $p < 0.05$.

Results:

GR and *FKBP5* expression were analyzed in the hippocampus and prefrontal cortex, which are thoroughly studied and well-established stress-responsive regions. As previously mentioned, the glucocorticoid receptor is abundantly expressed in those regions (Chao et al., 1989; Saánchez et al., 2000). The structures of dopaminergic pathways, the dorsal striatum and nucleus accumbens, also show a relatively high expression of the glucocorticoid receptor (Zoli et al., 1990) and are sensitive to stressful stimuli early in life (Schwabe and Wolf., 2013; Nylander et al., 2017; Enoch, 2011). The medial preoptic area is largely involved in the stress response pathway by receiving inputs from limbic structures and regulating the HPA axis (Myers et al., 2013). To our knowledge this study is the first to investigate the expression of GR and FKBP5 genes in response to MIA in the dorsal striatum, nucleus accumbens, and medial preoptic area.

Effects of stress and environment on FKBP5 mRNA expression

FKBP5 expression in the standard condition was significantly changed in the medial prefrontal cortex (p=0.007) (Fig. 1A), dorsal striatum (p=0.031) (Fig. 1B), nucleus accumbens (p=0.014) (Fig. 1C), dorsal hippocampus (p=0.028) (Fig. 1E), and ventral hippocampus (p=0.047) (Fig. 1F) with LPS-treated animals expressing higher levels compared to control animals. We also found a significant increase in *FKBP5* in the enriched environment with LPS-treated animals having significantly higher FKBP5 expression than the control animals (p= 0.027) (Fig. 1A). Contrarily to the enriched environment, the standard laboratory housing may contribute to the LPS-induced increased expression of *FKBP5* in those brain regions except in the medial prefrontal cortex. Neither stress nor the environment had a significant effect on *FKBP5* expression in the medial preoptic area (Fig. 1D). ANOVA analysis revealed a three-way interaction of treatment, housing, and sex on FKBP5 expression in the dorsal hippocampus ($F(1,35)=4.15$, $p=0.049$). Multiple comparisons revealed that control female rats expressed more *FKBP5* in the dorsal hippocampus than control males in the enriched environment ($p=0.035$) (see Supplementary Figure 1). This was the only sex difference observed in the data.

Effects of stress and environment on the glucocorticoid receptor expression

Within the enriched environment, the LPS-treated animals expressed significantly more GR in the medial preoptic area than the control animals (p=0.048) (Fig. 2D). There were no significant effects of MIA and EE in the expression of the glucocorticoid receptor in the medial prefrontal cortex, dorsal striatum, nucleus accumbens, and ventral and dorsal hippocampus.

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Discussion:

This study provides direct evidence of the alteration of FKBP5-GR mRNA expression in stress-sensitive regions of the rodent brain as a result of maternal infection during gestation, exposing the offspring to potentially detrimental dysregulation of FKBP5 and GR, two crucially and synergistically connected components to a proper adaptation to stress. Furthermore, the study explored a means of remediation of this dysregulation of *FKBP5* and *GR* mRNA by an enriched environment.

As expected, the expression of *FKBP5* significantly increased in almost all brain regions (prefrontal cortex, dorsal striatum, nucleus accumbens, dorsal and ventral hippocampus) with the exception of the medial preoptic area after exposure to early life stress. *FKBP5* is naturally upregulated by stress via the release of glucocorticoids. FKBP5 upregulation creates an ultra-short negative feedback loop wherein GR activity induces the transcription of FKBP5 which in turn reduces GR availability through its binding to the GR complex (Scharf et al., 2012, Vermeer et al., 2003). This negative feedback regulation by FKBP5 could correspond to the relatively low expression of GR in the same brain regions after the treatment with lipopolysaccharide. Our findings suggest an inadequate regulation of the negative feedback loop resulting in the disturbance of the HPA axis as well as the possibility for FKBP5-induced GR resistance, and a prolonged circulation of cortisol all characteristic of affective and anxiety disorders, namely MDD and PTSD (Holsboer., 2000; Pariante and Miller., 2001). An earlier study by Guidotti et al on the effects of CMS (chronic mild stress) on FKBP5 mRNA and protein expression as well as GR mRNA, protein, and cytosolic and nuclear expression in rodents showed similar results. They found that increased FKBP5 expression in CMS rats was associated with increased GR levels in the cytosolic compartment of ventral hippocampus and prefrontal cortex, which might be suggestive of an impaired ability of the receptor to translocate to the nucleus and activate GR-dependent transcriptional mechanisms (Guidotti et al., 2013). In consideration of the foregoing, lowering FKBP5 expression would rescue GR activity and confer resiliency to stress. Criado-Marrero et al (2017) remarkably demonstrated that low FKBP5 expression in the infralimbic cortex was indeed sufficient to both reduce fear acquisition and enhance fear extinction, suggesting that lower expression of FKBP5 in the ventral medial prefrontal cortex could contribute to resiliency to PTSD.

The dorsal striatum and the nucleus accumbens attract particular interest given our relatively limited understanding of the molecular responses to stress in these regions. The dorsal striatum and nucleus accumbens mediate cognition involved in stimulus-response or “habit acquisition” and the dopaminergic reward circuitry, respectively. It is well known that these functions have major implications in stress-related affective and psychiatric disorders. The mechanisms through which the stress axis interact with the dopaminergic system are still unclear, however a study by Nylander and colleagues postulated a central role for FKBP5 in early life stress-induced alteration in the mesolimbic reward circuitry leading to a propensity for alcohol abuse (Nylander et al., 2017). Studies in rodents have specifically showed that MIA disrupts dopaminergic activity (Luchicchi et al., 2016; Meyer et al., 2008). Our findings show MIA-induced dysregulation in FKBP5 expression in important structures of the reward system. This could suggest a potential mechanism through which maternal

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immune challenges could alter dopaminergic activity observed in people suffering from psychotic and depressive disorders. Much more is still to be learned about the effects of MIA in these brain regions in relation to FKBP5/GR expression and activity. It would be appropriate to conduct more thorough research using this model of MIA combined with behavioral tests to elucidate this possible mechanism.

The EE, despite its effect on *FKBP5* and *GR* expression being well documented in a very few studies (Sampedro-Pequiro et al., 2014; Tanichi et al., 2018; Candemir et al., 2019), did not produce enough conclusive evidence of its potential protective effect in the brain following MIA in our study. The stressed animals in the enriched environment showed a significant increase in *FKBP5* in the medial prefrontal cortex which was quite similar to the standard group. Perhaps EE does not alter FKBP5 expression in that brain region. Further work in this brain region is needed to reach a conclusive assessment. The medial preoptic area was the only brain region that was significantly affected by EE. The medial preoptic area directly participates in thermoregulation and cardiovascular control (Takahashi et al., 2001; Fassini et al., 2017) and is involved in the relay of the stress response pathway (Myers et al., 2013), key functions to an adaptive response to stress. We found that EE upregulated *GR* expression within the mPOA suggesting a potential mechanism of resiliency via the corticosteroid receptor. Surprisingly, we did not find a significant increase in hippocampal *GR* which is associated with cognition (S.Y.Lee et al., 2012) and which had been well reported to increase following EE (Olsson et al., 1994; Zhang et al., 2013b). Further research in this brain region with a possibly larger sample size is needed to have more statistically robust results considering the important of role of this region in paradigms of stress.

This study contains some limitations and further research should be conducted. Sweeping speculations cannot be made from these findings. In this study we only examined FKBP5 and GR expressions at a transcriptional level. The association between psychiatric disorders and these mRNA expression levels must be interpreted as correlative rather than causative. Measurements for mRNA and protein levels are both necessary to have full understanding of FKBP5 and GR activity. Furthermore, exploring GR target genes in response to the altered molecular expression induced by stress would deepen our understanding of the underlying mechanisms of psychiatric illnesses. Studies on the association of early life stress and the FKBP5-GR system action mainly investigate the vulnerability of different FKBP5 allelic variants to stressful experiences early in life and the implications of this interaction in the development of neuropsychiatric disorders (Criado-Marrero et al., 2019; Binder et al., 2008; Binder., 2009). This study does not specifically take FKBP5 polymorphisms into consideration, though genetic variability is an intrinsically important factor which could be at play in this type of investigation. It is also worth noting that males and females respond to stress and EE differently. While our data combined both sexes, potential differences between them were investigated. The only difference, following multiple comparison tests, was found in the dorsal hippocampus in which saline females had higher FKBP5 mRNA levels than saline male housed in the enriched environment. This could be reflective of an enriched environment-induced differential basal expression of *FKBP5* between the sexes. Note that the lack of robust results in reference to other regions could be explained by the possibility that the study was not sufficiently powered for a robust analysis of sex considering the sample size. Going forward, examining further the differences between the sexes with a

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larger sample size of males and females would be necessary to more adequately characterize sex differences and shed light on sex-specific effects of MIA and EE in those same regions.

This study adds to the growing literature and well documented effects of early life stress on the brain. The findings suggest that maternal infection during pregnancy alter the expression levels of FKBP5 and GR mRNA in different brain regions which result in the dysfunction of the HPA axis—characteristic of affective and mood disorders. Environmental enrichment promoted the expression of GR mainly in the mPOA which could suggest an adaptative mechanism in this area. Additional and supporting research is required to better delineate the potential protective effect of EE in other cortico-limbic regions affected by stress early in life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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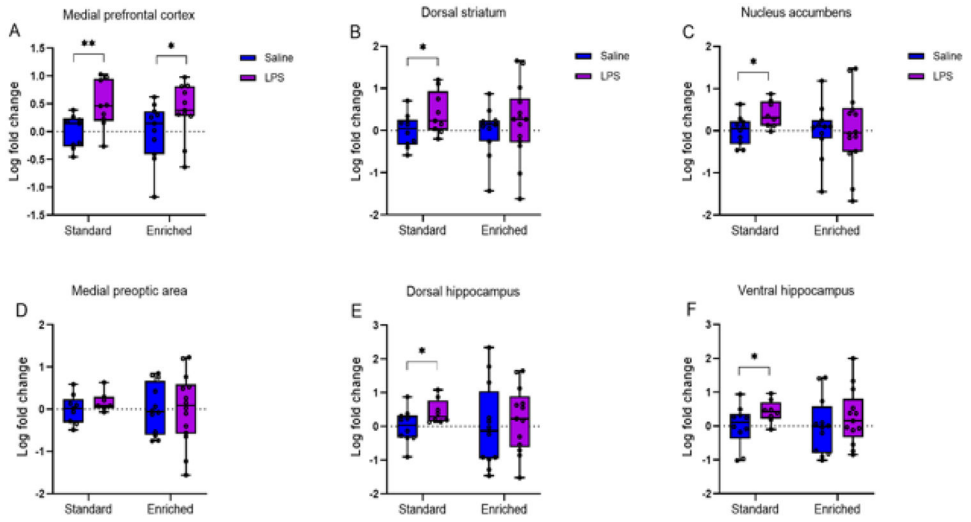

Figure 1.

RT-qPCR analysis of *FKBP5* expression change normalized to *Gapdh* in the prefrontal cortex, dorsal striatum, nucleus accumbens, medial preoptic area, dorsal hippocampus, and ventral hippocampus in control and stressed animal within a standard care environment vs. an enriched environment. (A) Stressed animals in the standard and enriched environment expressed more *FKBP5* in the medial prefrontal cortex than the unstressed animals (p=0.007) and (p=0.027) respectively. (B) Stressed animals in the standard environment expressed more *FKBP5* in the dorsal striatum than the unstressed animals (p=0.031). (C) Stressed animals in the standard environment expressed more *FKBP5* in the nucleus accumbens than the unstressed animals (p=0.014). (D) No differences in *FKBP5* expression in the medial preoptic area (p>0.05). (E) Stressed animals in the standard environment expressed more *FKBP5* in the dorsal hippocampus than the unstressed animals (p=0.028). (F) Stressed animals in the standard environment expressed more *FKBP5* in the ventral hippocampus than the unstressed animals (p=0.047). On the box and whisker plots, the "box" depicts the median and the 25th and 75th quartiles, the "whisker" shows the 5th and 95th percentile, n= 8-14 per treatment/housing. Individual data points are superimposed on the plots. * p<0.05, **p<0.01. FKBP5 log fold change gene expression data is presented in Supplementary Table 2.

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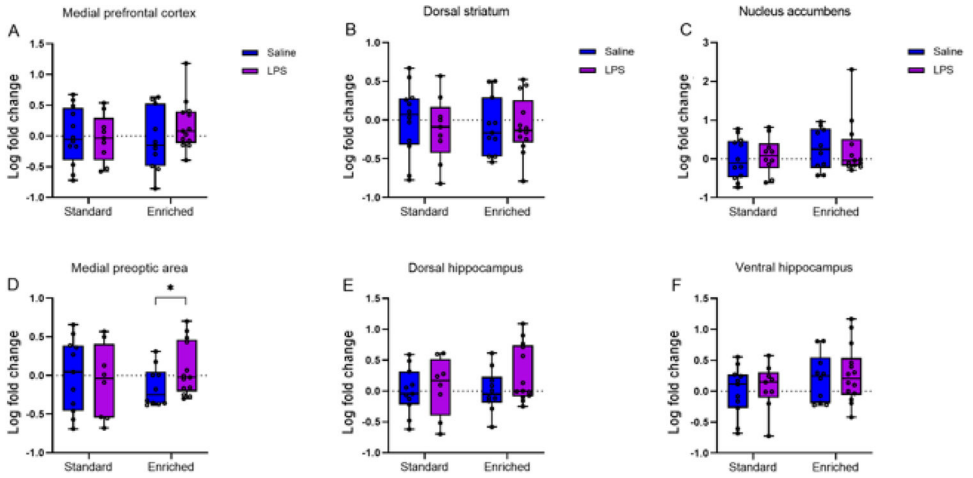

Figure 2.

RT-qPCR analysis of *GR* expression change normalized to *Gapdh* in the prefrontal cortex, dorsal striatum, nucleus accumbens, medial preoptic area, dorsal hippocampus, and ventral hippocampus in control and stressed animal within a standard care environment vs. an enriched environment. (A) No differences in *GR* expression in the medial prefrontal cortex ($p>0.05$). (B) No differences in *GR* expression in the dorsal striatum ($p>0.05$). (C) No differences in *R* expression in the nucleus accumbens ($p>0.05$). (D) Stressed animals in the enriched environment expressed more *GR* in the medial preoptic area than the unstressed animals ($p=0.0482$). (E) No differences in *GR* expression in the dorsal hippocampus ($p>0.05$). (F) No differences in *GR* expression in the ventral hippocampus ($p>0.05$). On the box and whisker plots, the “box” depicts the median and the 25th and 75th quartiles, the “whisker” shows the 5th and 95th percentile, n= 8-14 per treatment/housing. Individual data points are superimposed on the plots. * $p<0.05$. GR log fold change gene expression data is presented in Supplementary Table 2.

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Table 1:

Forward and reverse primer sequences

Primers	Forward	Reverse
Gapdh	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
GR	CCTCAGCGCTCTTGGAAATTA	CCACCCTTCTGTCCTGTTTATG
FKBP5	GCAGTCGGAGTGAGTTATCTTC	CTAGGACAAGAGCAAGCCTAAG