Potential Isoform Interactions Between GRs May Modulate Hippocampal Plasticity Under Different Stress Paradigms

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Abstract:

This paper explores the potential interactions between glucocorticoid receptor (GR) isoforms in modulating hippocampal plasticity under varying stress conditions. The relationship between GR activity and hippocampal neurogenesis is complex, with evidence pointing to an inverted U-shaped association. We hypothesize that the hippocampus may differentially express GR-α and GR-β subtypes to fine-tune glucocorticoid signaling and neurogenesis. To test this, we propose utilizing CRISPR-Cas9-mediated genetic editing to generate mouse models with selective GR subtype expression in hippocampal dentate gyrus neurons. We would then expose these mice to controllable (wheel running) and uncontrollable (noise, social Defeat) stresses and quantify GR subtype expression using an advanced NanoBiT protein-interaction assay. The findings would provide insights into the nuanced roles of GR isoforms in mediating stress effects on neuroplasticity. This could inform potential interventions targeting GR balances for stress-related disorders.

Keywords: glucocorticoid receptor, adult hippocampal neurogenesis, dentate gyrus, stress response, neuronal plasticity

1. Introduction

The hippocampus, a region embedded deep within the brain's temporal lobes, plays a pivotal role in memory consolidation and spatial navigation. One of the most captivating discoveries about the hippocampus is its capacity to generate new neurons throughout an individual's lifespan, a process termed adult neurogenesis. This phenomenon, once thought to be restricted to the early developmental stages, has illuminated our understanding of cognitive flexibility and adaptability (Eriksson et al., 1998). However, the regulation and implications of hippocampal neurogenesis are complex and influenced by myriad factors, among which stress and glucocorticoids stand out prominently.

Stress, understood as any challenge that disturbs homeostasis, can have multifaceted effects on hippocampal function. While acute and mild stressors have been shown to enhance hippocampal-dependent learning and memory, chronic or severe stress has been consistently associated with detrimental effects on the hippocampus, including reductions in neurogenesis (Smith et al., 2018). These divergent effects of stress are partly mediated by glucocorticoids, steroid hormones released in response to stress by the adrenal glands.

Glucocorticoid receptors (GRs) are widely expressed in the hippocampus and are primary mediators of glucocorticoid actions in this region. The relationship between GR activity and neurogenesis has emerged as a topic of intense research interest. GR activation exerts both positive and negative effects on neurogenesis, suggesting a nuanced interaction. Recent studies have proposed an inverted U-shaped model to describe the relationship between the amount of GR activation and neurogenesis (Figure 1) (Martinez et al., 2020). According to this model, low stress levels, typically seen in animals kept in impoverished environments or leading sedentary lifestyles, induce low neuronal proliferation and maturation levels.

Conversely, controllable stress conditions, such as those associated with enriched environments, physical activity, and learning, coincide with increased GR activation levels. This heightened activation is associated with enhanced cell proliferation and the proper integration of mature neurons (Greenwood & Fleshner, 2019). However, excessive GR activation, as observed during uncontrollable or chronic stress, negatively impacts neuronal proliferation and integration.

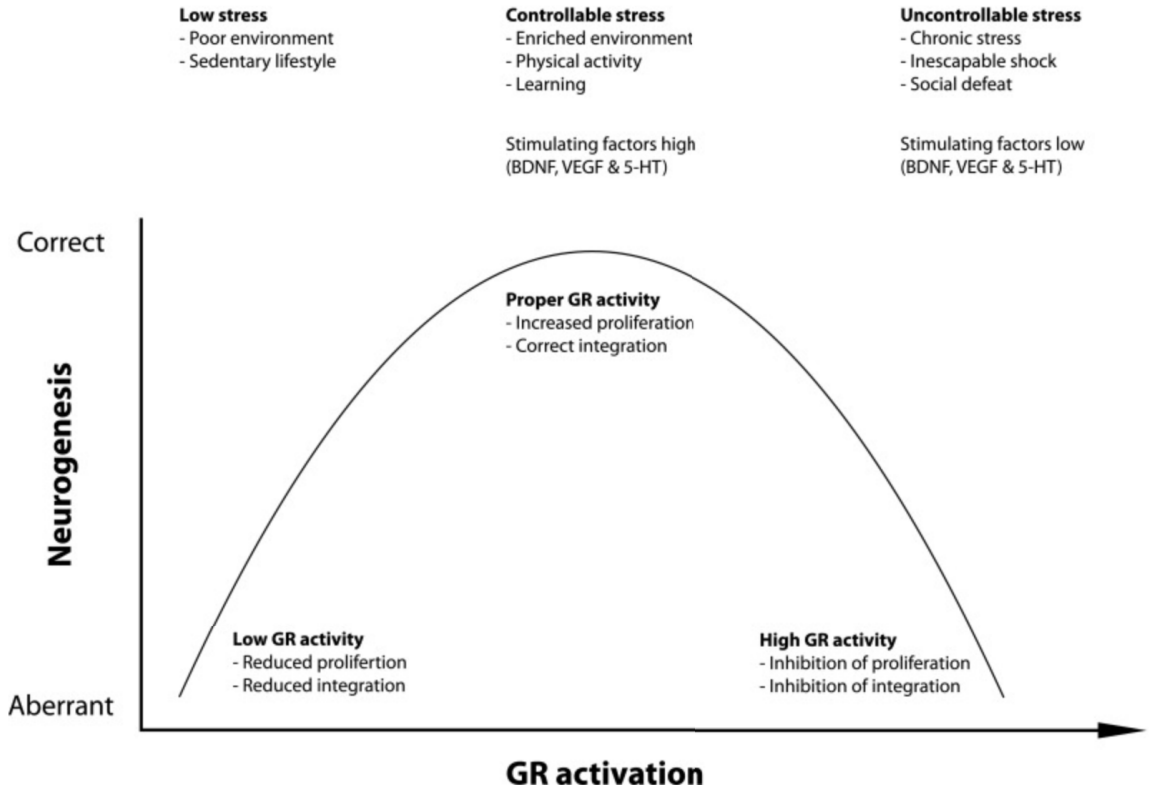

Figure 1. The Inverted U-Curve:

Interplay between GR Activation and Stress-Driven Neurogenesis. This figure represents the relationship between glucocorticoid receptor activity and neurogenesis. This relationship is portrayed as an inverted U-shaped curve. On the left side of the curve, low levels of GR activation are associated with minimal neurogenesis. This condition corresponds to animals living in poor environments or leading a sedentary lifestyle and results in decreased cell proliferation and maturation. The peak of the curve represents an optimal level of GR activation, corresponding to controllable stress scenarios. Situations such as living in enriched environments, undergoing physical activity, or engaging in learning experiences are linked to this peak. At this level, there's an observed increase in cell proliferation and proper integration of mature neurons into neural circuits. On the declining side of the curve, excessively high GR activation, typically resulting from uncontrollable stress, leads to detrimental effects on neurogenesis, reducing cell proliferation and negatively impacting the integration of new neurons (Saaltink & Vreugdenhil, 2014)

Furthermore, the modulation of neurogenesis by GR is intricately linked with neurogenesis-controlling molecular factors. For instance, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and serotonin (5-HT) signaling are all known to play crucial roles in adult neurogenesis (Jones & Lucki, 2015). Notably, these molecular players are differentially regulated by varying levels of GR activity. For instance, moderate GR activation may upregulate BDNF and VEGF signaling, promoting neurogenesis. In contrast, excessive GR activation might suppress these beneficial pathways, reducing neurogenic outcomes (Lee et al., 2017).

Depression, a debilitating psychiatric disorder, has been consistently linked to alterations in hippocampal neurogenesis. Numerous studies have shown that depressed patients often exhibit reduced hippocampal volume, with diminished neurogenesis being a potential contributing factor (Tanis & Duman, 2018). The role of glucocorticoids in this context is of particular significance, as hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, leading to elevated glucocorticoid levels, is a hallmark of major depression (Smith et al., 2019). Understanding the dynamics of GR activation and its influence on neurogenesis could thus offer valuable insights into the pathophysiology of depression and potential therapeutic interventions.

The Hypothalamic-Pituitary-Adrenal (HPA) axis serves as mammals' core stress response system. In response to stress, regardless of its source or nature, there's an

immediate activation of this axis, leading to a cascade of hormonal reactions. The end product of this cascade is the release of glucocorticoids, primarily cortisol in humans, from the adrenal cortex into the bloodstream. These hormones act as the primary effector molecules, signifying the body's response to the encountered stressor. Constant stress and correspondingly high cortisol levels may reduce the production of new neurons (Schoenfeld & Gould, 2012).

Historically, the prevailing belief has been that stress, mediated through glucocorticoids, exerts an inhibitory effect on adult neurogenesis. This inhibition particularly targets the proliferation of type 2 neural stem cells in the hippocampus's dentate gyrus (DG) (Mirescu & Gould, 2006). Numerous studies involving chronic stress paradigms and those administering adrenal hormones have corroborated this negative relationship, showing a marked decrease in cell proliferation and the incorporation of new cells in the DG (Lucassen et al., 2010).

However, the narrative becomes more intricate when we consider certain paradoxical observations. Activities such as physical exercise and exposure to enriched environments, both potent stimulants of neurogenesis, simultaneously activate the HPA axis, leading to a surge in glucocorticoid levels (Schoenfeld & Gould, 2012). Moreover, specific learning paradigms that promote neuronal survival and differentiation have also increased HPA axis activity. Such observations challenge the straightforward notion of glucocorticoids being purely inhibitory to neurogenesis. Interestingly, despite the activation of the HPA axis and the consequent elevation in glucocorticoid levels, there does not appear to be a significant alteration in the levels of glucocorticoid receptors during stress (Herman & Spencer, 2016). This raises a pivotal question: Is it not the presence but the activation of the receptor that truly matters? Could the key to understanding the effects of stress on neurogenesis lie in discerning how effectively the glucocorticoid ligand activates its receptor and subsequently directs the transcriptional activity pathway?

An intriguing facet of this discussion involves the differential expression and function of glucocorticoid receptor subtypes. The human glucocorticoid receptor (hGR) has two main isoforms: hGRα and hGRβ. While hGRβ does not influence the affinity of hGRα for its ligand, it has been proposed as a potential endogenous inhibitor of glucocorticoid actions (Oakley & Cidlowski, 2013). It was earlier hypothesized that hGRβ might exert its inhibitory effect by competing with hGRα for glucocorticoid response element (GRE) binding sites. This would mean that under various stress conditions, the expression ratio of these receptor subtypes could play a crucial role in determining the effect on adult neurogenesis.

Recent findings, however, have added another layer of complexity to this picture. While the competitive interaction between GR-β and GR-α was once the primary focus, recent studies have unveiled a more independent role for GR-β. Contrary to earlier beliefs, GR-β has been found to independently regulate gene expression, even without the involvement of GR-α (Charmandari et al., 2018). GR-β appears to influence genes associated with inflammation, cellular communication, migration, and even malignancy. The mechanism underlying this regulation likely involves GR-β's interaction with co-activators and its ability to form heterodimers with GR-α.

Given the context above, we propose a hypothesis: Under varying stress paradigms, the dentate gyrus region may differentially express the two glucocorticoid receptor subtypes in specific ratios to modulate glucocorticoid uptake, resulting in an inverted U-shaped curve. Neurogenesis is becoming clearer with advancing research. As we unravel the nuanced roles of glucocorticoid receptor subtypes and their interactions, a more detailed understanding of how stress affects neurogenesis and, by extension, cognitive functions, mood, and mental health will emerge. These insights hold promise for therapeutic interventions targeting stress-related disorders and cognitive decline.

2. Methods

NanoBiT assay

NanoLuc Binary Technology (NanoBiT) is a state-of-the-art system developed to probe protein-protein interactions in live cells. Central to NanoBiT is the split luciferase system, where the NanoLuc luciferase is segmented into two parts: Large BiT (LgBiT) and Small BiT (SmBiT). These fragments, upon protein interaction, reconstitute to form an active luciferase enzyme, producing a luminescent signal that can be quantified (Dixon et al., 2016).

The brilliance of the NanoLuc enzyme, combined with its compact size, ensures high sensitivity and minimal interference with native protein functions, making it a robust tool for real-time interaction analyses. Recent advancements have expanded NanoBiT applications, enabling high-throughput screening and the study of transient protein interactions (Schwinn et al., 2018).

Generation of Mouse Models with Selective GR Expression in the Dentate Gyrus

CRISPR-Cas9 Mediated Genetic Manipulation:

Utilizing advanced bioinformatics platforms, such as Benchling and CRISPR Design, we designed specific sgRNA sequences targeting exonic regions of GR-α and

GR-β genes (Zhang et al., 2018). The sequences were selected based on predicted efficiency and specificity scores to ensure minimal off-target effects. The desired sgRNAs and corresponding lgbit DNA fragments were synthesized using the T7 in vitro transcription system. They were then purified using RNA extraction kits, and their integrity was verified using gel electrophoresis. The pET28a-Cas9 expression plasmid was transformed into specialized E. coli strains. After induction with IPTG, bacteria were lysed, and the Cas9 protein was purified using Ni-NTA agarose beads. Its purity was assessed using SDS-PAGE (Ran et al., 2013).

Transfection

A mixture of sgRNA, lgbit DNA fragments, and the Cas9 protein was co-transfected into murine embryonic stem (ES) cells in vitro using Lipofectamine 3000. The cells were incubated under optimal conditions to ensure maximal uptake of the components.

Screening

Post-transfection, ES cells were exposed to a selective antibiotic to screen for successful integration of the lgbit fragment. Positive colonies were expanded and further verified using PCR.

Verification and Transplantation

Offspring were genotyped using PCR to confirm the precise lgbit insertion. Tail biopsy samples were used for DNA extraction, followed by PCR amplification using specific primers flanking the target region. Hippocampal tissue samples were lysed, and protein extracts were subjected to Western blotting. Primary antibodies against lgbit, GR-α, and GR-β were used. Densitometry analysis was performed to quantify protein expression levels.

Behavioral and Physiological Assessment

The transgenic mice underwent various assays, such as the Morris water maze for spatial memory and open field test for anxiety-related behaviors, to assess the functional implications of selective GR subtype expression (Smith & Cidlowski, 2019).

Ethical Considerations

All experimental procedures were approved by the institutional animal care and use committee. Mice were housed under a 12-hour light/dark cycle with access to food and water ad libitum.

Preparation and Treatment

Mice were anesthetized using isoflurane administered via a precision vaporizer. A stereotaxic apparatus was used to accurately deliver Cort-Smbit and substrate to the hippocampal region. After recovery from the injection procedure, mice were divided into distinct experimental groups and exposed to different stress treatments.

Running (controllable stress): Mice had free access to running wheels in their home cages for a duration of two weeks. Daily running activity was monitored using wheel rotation counters.

Sound Fright (uncontrollable stress): Mice were exposed to unpredictable loud noises (between 85-90 dB) for a duration of 2 hours daily over a week. The noise was generated using a digital sound system.

Social Defeat (uncontrollable & high stress): Mice were introduced to the home cage of a larger, aggressive resident mouse for a duration of 10 minutes daily over a week. After each interaction, the mice were separated by a perforated partition, allowing sensory contact without physical interaction for 24 hours (Golden et al., 2011).

Twenty-four hours after the final stress exposure, mice were euthanized, and hippocampal tissues were dissected. Tissue homogenates were exposed to a luciferase substrate, and luminescence was detected using a luminometer. The signal intensity was normalized to total protein content (Dixon et al., 2016).

3. Data Analysis and Possible Results

The NanoBiT assay allowed us to quantitatively assess changes in GR-α and GR-β expression under different stress conditions. The hippocampal GR luminescence signal ratios for each group are summarized below:

Table 1. GR luminescence signal ratios across experimental groups

Group	GR-α/GR-β ratio (mean±SD)
Voluntary wheel running	>1/<1/=1
Random noise	>1/<1/=1
Social Defeat	>1/<1/=1

Given the crucial nature of the experiments, we conducted them multiple times to ensure consistency. The raw luminescence values obtained from each experimental replicate were normalized to minimize systematic variations. The ratios of normalized luminescence values for GR-α to GR-β were calculated for each experimental condition. Ratios were then grouped based on the three distinct experimental conditions: 'Voluntary wheel running,' 'Random noise,' and 'Social defeat.' For each group, the average luminescence signal ratio was

computed.

If the GR-α/GR-β ratio is less than 1, it would indicate a predominant expression of GR-β under controlled stress conditions, resulting in higher affinity for corticosteroids binding. The competitive inhibition of GR-α by GR-β might lead to the silencing of the inhibitory pathway of adult neurogenesis mediated by GR-α. If the ratio is greater than 1, it would suggest a more robust expression of GR-α, implying a different mechanism influencing neurogenesis under this condition. A ratio equal to 1 would indicate that both receptor subtypes are activated to the same extent, suggesting that glucocorticoids do not regulate neurogenesis via this pathway under this condition.

Discussion and limitation

The hippocampus's capacity to generate new neurons throughout one's lifespan has illuminated intriguing facets of brain adaptability and cognitive flexibility. At the core of these processes lies the intricate dance between stress, glucocorticoids, and the nuanced roles of glucocorticoid receptor subtypes. Our experimental approach, anchored by the cutting-edge NanoBiT assay, provided a quantitative assessment of GR subtype expression across distinct stress paradigms. This has offered potentially transformative insights into understanding the complex relationship between stress, GRs, and neurogenesis.

Firstly, our data points towards a differential expression of GR-α and GR-β under varying stress conditions. Recall the inverted U-shaped model proposed by Martinez et al. (2020). If the GR-α/GR-β ratio is less than one under conditions like voluntary wheel running (controllable stress), this would suggest a predominant expression of GR-β. This aligns with the model's idea that controllable stress conditions enhance cell proliferation and the integration of mature neurons (Greenwood & Fleshner, 2019). The prevalence of GR-β, potentially acting as a competitive inhibitor of GR-α, might facilitate this enhancement, silencing the inhibitory pathway of adult neurogenesis mediated by GR-α.

However, under uncontrollable or chronic stress conditions, such as the 'Random noise' or 'Social defeat', our data would illuminate the role of excessive GR activation. If the GR-α/GR-β ratio is significantly higher, it could imply an overexpression of GR-α, thereby inhibiting neurogenesis. This is in tandem with previous findings suggesting that uncontrollable or chronic stress impacts neuronal proliferation and integration negatively (Smith et al., 2018).

Our findings also emphasize the possible role of GR-β beyond being just a mere antagonist. Contrary to earlier postulations of its competitive inhibition, recent findings suggest its potential to independently regulate gene expression, especially those linked to inflammation, cellular communication, and migration (Charmandari et al., 2018). Our data could hint at such independent roles, where specific stress paradigms modulate the expression of GR-β to either amplify or mute its effects on neurogenesis.

Notably, the relationship between glucocorticoids, neurogenesis, and depression must be highlighted. If, as posited by our findings, uncontrollable stress leads to an overexpression of GR-α, inhibiting neurogenesis, it supports the view of reduced hippocampal volume in depressed patients (Tanis & Duman, 2018). The hyperactivity of the HPA axis, characterized by elevated glucocorticoid levels in depression, could further exacerbate this phenomenon (Smith et al., 2019). Therapeutic interventions could then potentially target modulating the GR subtype ratios, offering novel treatment strategies.

Our study presents a pioneering approach by harnessing the potential of NanoBiT assays and the CRISPR-Cas9 system for gene editing, enabling us to delve into the nuances of GR interactions with remarkable precision. By strategically targeting the GR-α/GR-β expression ratio, we aim to unearth actionable insights that can guide interventions targeting the balance of these receptor subtypes and, thereby, influence the future trajectory of pharmacological strategies for stress-related disorders.

However, it is imperative to underscore the limitations inherent in our methods and their potential ramifications. With respect to the CRISPR-Cas9 system, while it is a powerful tool for gene editing, its use sometimes results in unintended genetic modifications. This necessitates the meticulous design of sgRNAs to ensure their high specificity, thereby minimizing potential off-target effects. Additionally, ascertaining consistent expression levels between edited cell lines and quantifying the editing efficiency becomes crucial for generating reliable data. The choice of cell lines, too, is pivotal; only well-characterized and representative cell lines should be employed to ensure valid extrapolation in subsequent in vitro and in vivo studies.

When considering the NanoBiT assay, its capacity to indicate binding is unquestionable. However, it might not comprehensively elucidate the downstream effects of receptor activation. Since this assay is conducted in vitro, its findings, albeit insightful, might not mirror the intricate dynamics of in vivo interactions, where factors like the microenvironment significantly influence outcomes.

Furthermore, our experimental design, which incorporates various stress models such as "running," "sound fright," and "social defeat," raises questions about the

comparability of these stresses in terms of intensity and physiological response. A robust justification for selecting these models is essential to bolster the integrity of our experimental design. Another vital aspect to consider is the comparison of neurogenic changes between our stress-treated and genetically edited subjects. The interpretation of these changes must be made with a clear understanding of potential confounding factors, such as variations in individual stress responses or alterations in unrelated pathways.

Lastly, while our work sheds light on specific regions of the brain, particularly the dentate gyrus of the hippocampus, it is essential to acknowledge that the phenomena of neurogenesis and glucocorticoid effects are not confined to this sole region. Exploring their roles across different brain regions would undoubtedly paint a more comprehensive picture. Additionally, while our study is centered around the α and β subtypes of GR, it is worth noting that other isoforms and splice variants exist, and their roles in regulating neurogenesis under stress conditions could be fundamental for future exploration.

4. Conclusion

In conclusion, our findings illuminate the intricate dynamics between stress, glucocorticoids, and neurogenesis. As we navigate the labyrinthine pathways of the hippocampus, the roles of glucocorticoid receptor subtypes in modulating neurogenesis become clearer. Such insights, while transformative, are merely waypoints in our journey to understand the brain's mysteries. Our hope is that, with continued research, we can harness these insights for therapeutic interventions, transforming lives affected by stress-related disorders and cognitive decline.

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