

## REVIEW ARTICLE

# How Stress Gets Under the Skin: Early Life Adversity and Glucocorticoid Receptor Epigenetic Regulation

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**Abstract:** Early life adversity is associated with both persistent disruptions in the hypothalamic-pituitary-adrenal (HPA) axis and psychiatric symptoms. Glucocorticoid receptors (GRs), which are encoded by the *NR3C1* gene, bind to cortisol and other glucocorticoids to create a negative feedback loop within the HPA axis to regulate the body's neuroendocrine response to stress. Excess methylation of a promoter sequence within *NR3C1* that attenuates GR expression, however, has been associated with both early life adversity and psychopathology. As critical regulators within the HPA axis, GRs and their epigenetic regulation may mediate the link between early life adversity and the onset of psychopathology. The present review discusses this work as one mechanism by which stress may get under the skin to disrupt HPA functioning at an epigenetic level and create long-lasting vulnerabilities in the stress regulatory system that subsequently predispose individuals to psychopathology. Spanning prenatal influences to critical periods of early life and adolescence, we detail the impact that early adversity has on GR expression, physiological responses to stress, and their implications for long-term stress management. We next propose a dual transmission hypothesis regarding both genomic and non-genomic mechanisms by which chronic and acute stress propagate through numerous generations. Lastly, we outline several directions for future research, including potential reversibility of methylation patterns and its functional implications, variation in behavior determined solely by *NR3C1*, and consensus on which specific promoter regions should be studied.

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## 1. INTRODUCTION

Early life represents a critical period for the development of the neuroendocrine stress system and hypothalamic-pituitary-adrenal (HPA) axis functioning. While it may seem obvious that chronic early life stress - such as sexual abuse, neglect, and physical abuse - has negative psychosocial and physical repercussions [1-4], researchers have begun to demonstrate that early life adversity also extends to the core building blocks of life: DNA [5]. When a healthy individual confronts a stressor, the HPA axis becomes activated: the hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary gland to secrete adrenocorticotropic hormone (ACTH), which then stimulates the adrenal gland to release cortisol into the bloodstream. Cortisol in turn prepares the body's fight or flight response to manage the stressor. Since cortisol is a glucocorticoid, its binding with glucocorticoid receptors (GRs) across the body - predominantly in the hippocampus, the brain region with the highest concentration of GRs

[6, 7] - creates a negative feedback loop that inhibits the HPA axis and subsequently diminishes the body's neuroendocrine response to stress [8, 9].

Growing evidence indicates that the number of GRs is attenuated in people with a history of early life stress [10-13]. This reduces the ability of GRs to regulate the negative feedback loop, generating persistent HPA axis dysregulation and heightened cortisol levels in response to stressors [14]. Accompanying these alterations are sustained increases in CRH and ACTH levels, reflecting systemic modifications to the HPA axis [15, 16]. These effects may be mediated, in part, by transcriptionally silencing the promoter region within *NR3C1* via the epigenetic process of DNA methylation. Since *NR3C1* is the gene that codes for GRs, it plays a critical role in mediating the stress response.

GRs have a low affinity for cortisol and thus predominantly respond to higher and persistent levels of cortisol, which correspond to chronic and prolonged stressors [17, 18]. Extreme levels of stress, and the subsequent downregulation of GRs via epigenetic mechanisms, have long-term and potentially devastating implications for the body's stress regulatory mechanisms [19]. This downregulation may initially serve a compensatory function in response to the acute and chronic stress. However, over time, decreased GR ex-

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pression would exaggerate one's stress response. In fact, childhood maltreatment and the subsequent alteration of the HPA axis, especially with regards to GRs, have been robustly implicated in the development of numerous stress, mood, substance abuse, and other psychological disorders later in life, including borderline personality disorder, major depressive disorder and schizophrenia [20-23]. Thus, decreasing the expression of GRs is one mechanism through which early adversity sensitizes individuals to psychological vulnerabilities and exerts its deleterious effects on development (see Graphical Abstract for a conceptual framework).

The goal of the present paper is three-fold. First, we highlight how early life stress gets under the skin to initiate long-lasting alterations in the stress response system and predispose individuals to psychopathology through altering *NR3C1* expression. This paper will comprehensively synthesize the extant literature from both rodent and human studies and discuss developmental implications stemming from early life stress. Second, we explicate how the effects of early life adversity on stress regulatory systems may extend into future generations. While previous literature has separately analyzed genomic and nongenomic methods of epigenetic transmission, we discuss a dual intergenerational transmission mechanism of transmitting vulnerabilities in the stress response system. In doing so, we will also elucidate the role that epigenetic transmission has on psychopathology and the propagation of phenotypic vulnerabilities. Third, we will discuss the critical areas of future research to more fully understand *NR3C1* epigenetic regulation, the HPA axis, vulnerabilities for psychopathology, and intergenerational transmission of epigenetic marks and the aforementioned vulnerabilities.

## 2. GRs AND THE NR3C1 GENE

The gene that codes for GRs, *NR3C1*, is located on chromosome 5 and is expressed in nearly every cell in the human body [24, 25]. As steroid receptors, GRs are located cytoplasmically, and upon binding with cortisol or other glucocorticoids, exert their regulatory effects in the HPA axis stress pathway through altering gene expression [26]. Proper GR expression, which relies on the accessibility of the *NR3C1* promoter region, is thus necessary for proper negative feedback signals to regulate the stress response within homeostatic parameters.

The *NR3C1* gene contains nine exons, which are regions of base pairs that are either translated into GRs or regulate their expression and cellular processing [7]. The regulatory region consists of multiple first exons, each controlled by its own promoter, which regulates the gene's transcription [27]. Although there are seven alternative exon splicing loci within the first exon, research has focused on the methylation patterns and clinical relevance stemming from exon 1<sub>F</sub>, the human homolog of the rat exon 1<sub>7</sub>. This exon is heavily expressed in the hippocampus and peripheral biology, including the immune system [28, 29]. This promoter sequence, positioned directly at the upstream of the coding exons in *NR3C1*, contains a dense population of cytosine-guanine dinucleotide (CpG) sites and a canonical region to which binding of the transcription factor nerve growth factor-induced protein A (NGFI-A) enhances transcription and

the subsequent expression of GRs [11]. As a result of early life adversity, methyl groups may be deposited on the CpG sites in the NGFI-A binding region, thereby inhibiting transcription factor binding and gene transcription [30, 31]. Indeed, increased methylation of *NR3C1* is associated with decreased NGFI-A binding, mRNA expression, and GR expression in the brain, peripheral tissues, and blood [10, 11, 32-34].

Cytosine methylation is a stable modification that can continue to mediate long-term behavioral and physiological alterations in adulthood. A study that assessed the stability of a broad range of epigenetic marks over 14,000 genes found that the effects of stress experienced in early life were still detectable in the epigenome over a decade later [35]. With regard to *NR3C1* methylation, the epigenetic marks may be stable enough to last throughout life in both rats and humans and correspond to phenotypic variations and vulnerabilities [11, 12, 32, 36].

## 3. EARLY LIFE STRESS AND EPIGENETIC MODIFICATIONS IN RATS AS A MODEL FOR HUMAN STUDIES

Studies analyzing the epigenetic bridge between early life experiences and their long-term effects on HPA axis responsiveness were first conducted in rats [37]. This laid the groundwork for translating these studies into human research. Precise experimental control in rat studies grants researchers the capability to isolate the effects of modifying GR expression on behavioral alterations, and to also disentangle the effect of genes from environment. The rat model thus provides insight into the mechanisms by which stress gets under the skin.

When mother rats engaged in more pup licking, grooming, and arched-back nursing (LG-ABN) behaviors, their offspring exhibited decreased methylation in the NGFI-A binding site in exon 1<sub>7</sub> promoter in the hippocampus within the first week of birth [32]. These offspring not only had increased hippocampal GR expression, but also demonstrated better control over their glucocorticoid feedback system as adults, as indicated by reduced ACTH and cortisol levels and CRH mRNA transcripts [38].

However, in pups whose mothers did not express these affectionate behaviors, the 1<sub>7</sub> region in the hippocampus became significantly more methylated, which was associated with decreased NGFI-A binding [39]. These epigenetic changes also remained stable into adulthood and were associated with altered HPA axis responsivity, as indicated by elevated corticosterone levels following a stressor [32, 40]. This suggests that early life adversity perturbs rat pups' ability to regulate their physiological responses to stress *via* altered epigenetic states. However, if the rat pups were cross-fostered such that the mothers with different nurturing styles were exchanged to raise the rat pups from a different biological mother, then the epigenetic patterns and neurodevelopmental responses to stress were reversed [32, 41]. Since cross-fostering controls for potentially confounding genomic variations by only changing the nurturing style of the surrogate mothers that raised the cross-fostered rat pups, it suggests a strong influence of environmental factors on the epigenetic control of *Nr3c1*.

Significant decreases in *Nr3c1* mRNA levels in the hypothalamus and limbic regions were also observed in newborn rats just 24 hours after maternal deprivation, indicating that these effects may be rapidly initiated [42]. Maternal separation has also been associated with hypermethylation of the promoter region within *Nr3c1* [32, 43, 44]. These two observations provide a functional link between promoter methylation and GR expression since promoter methylation would restrict NGFI-A binding to initiate *Nr3c1* transcription and produce GR mRNA transcripts. In accordance with *Nr3c1* promoter methylation and the downregulation of a critical component in the HPA axis negative feedback system, consistent disruptions in maternal interactions in rats have also been shown to increase CRH expression and HPA axis response [45]. However, removing the differences in hippocampal GR binding capacity through neonatal handling eliminates any differences in HPA axis responses to stress in adulthood that were caused by early life adversities [46]. Thus, while a loving maternal environment can foster the development of a healthy physiological response system to stress, poor maternal care can result in aberrant HPA axis control and persistently elevated cortisol levels due to blunted GR expression.

Interestingly, environmental influences stemming from as early as prenatal factors can also influence methylation and developmental patterns observed in offspring [47-49]. In accordance with this fetal programming hypothesis, rats' maternal stress during pregnancy has been shown to be associated with disrupted stress responsivity, depressive behaviors, and altered GR methylation and expression in the hypothalamus and amygdala in the offspring [50, 51]. In a separate study, maternal stress during pregnancy directly modified subsequent maternal care of rat pups to induce less licking and grooming behavior [52]. This maternal behavioral alteration was associated with increased anxiety levels and HPA axis activity in adulthood in the offspring, which is consistent with the findings from Weaver *et al.* [32].

Furthermore, a direct link between a modified epigenetic state and phenotypic alterations in HPA axis stress responsivity has been established. Administration of a histone deacetylase inhibitor to decompact the dense chromatin state programmed by low LG-ABN mothers restored NGFI-A binding to exon 1<sub>7</sub>, GR expression, and HPA axis activity to similar levels of those present in rats raised by high LG-ABN mothers [32]. Similarly, induced hypermethylation of the 1<sub>7</sub> promoter reversed GR expression and HPA axis response to stress in the adult brain of rats raised by high LG-ABN mothers, thereby providing a direct and potentially reversible link between environmental programming and epigenetic status [53]. Since this effect was observed in adult rats, this suggests that reversibility of the aberrant phenotype is not age dependent, but rather dependent upon the epigenetic state.

However, the histone deacetylase inhibitor used to alter epigenetic state in these studies was broadly acting and may have influenced other genes involved in the stress response system besides just *Nr3c1* [32]. With current techniques, it is difficult to differentially modify methylation patterns in specific regions of the genome. Experiments involving RNA silencing or exogenous expression of GR promoters could

serve as proxies, but these do not perfectly represent a modified epigenetic state. However, extensive cross fostering studies at various stages along the developmental pathway in rats could reveal the extent to which age is a contributing factor in modulating the reversibility of environmental programming of the epigenetic state. Moreover, these studies could also elucidate the precise sensitivity window for inducing epigenetic changes that are associated with stable alterations in stress reactivity and uncover if reversing these phenotypic variations is solely dependent upon epigenetic state at each developmental stage.

Early life stress is therefore significantly implicated in rat pups' developmental outcomes, and research suggests that it is also involved in the intergenerational transmission of dysregulated stress response systems. The mechanisms of intergenerational transmission likely include both nongenomic and genomic components.

Evidence for a nongenomic mechanism comes from studies analyzing the developmental outcomes of rat pups and their subsequent parenting styles as adults. As compared to adult offspring of high LG-ABN mothers, those raised by low LG-ABN mothers are behaviorally more fearful and exhibit exacerbated stress responses, which influence their parenting style [40, 52, 54]. The offspring of mothers who experienced gestational stress perpetuated their aberrant nurturing behaviors and thus raised their offspring in a similar, stress-inducing environment even when the original stressor in the first generation was removed [55]. Thus, the effects of gestational stress were propagated across multiple generations through alterations in maternal care, not necessarily through epigenetic mechanisms [56]. Moreover, induced maternal stress following parturition also resulted in altered maternal behavior toward the offspring, with similar developmental implications [55].

Evidence for a nongenomic method of intergenerational transmission also comes from cross fostering studies. When the pups of either low or high LG-ABN mothers were cross fostered, they adopted the mothering style of their adoptive parents and not their biological parents as adults [38, 41, 45]. Since all these studies examined differences in maternal rearing behaviors, an interesting area of future research would be to examine the potential contributions of paternal influences. If there is a robust paternal effect, then the offspring of male adult rats raised by either high or low LG-ABN mothers should also display differences in parenting styles stemming from paternal influences after controlling for maternal contributions.

However, it is necessary to also consider the genomic component in mediating the multi-generational transmission of altered stress responses, especially since dysfunctional maternal behavior induces epigenetic changes in their offspring that alter their capacity to handle stress [32, 41]. Thus, with regards to the cross fostering studies [41], it is possible that epigenetic processes mediated why low LG-ABN mothers raised low LG-ABN pups and *vice versa*. Upon being cross fostered to a low LG-ABN mother, rat pups may be conferred with an epigenetic scar that alters long-term HPA axis functioning [32]. These epigenetic modifications in turn may mediate the consequent transmission of maternal behavior since *Nr3c1* promoter methylation mediates the devel-

opment of anxiety-like behavior and since maternal behavior is linked to maternal mood [32, 52]. Indeed, cross fostering between low and high LG-ABN mothers not only changes the subsequent mothering styles that the pups adopt, but it also reverses the epigenetic patterns [32, 41], (for review of rodent studies [57]).

Thus, it is not entirely clear to what extent maternal care and differences in stress reactivity can be transmitted without the corresponding epigenetic signatures. Accumulating evidence suggests that both nongenomic and genomic mechanisms contribute to this process, but unless cross fostering studies are conducted in which the epigenetic alterations in the rat pups are held constant or otherwise controlled for, disentangling their individual contributions remains elusive and difficult.

#### 4. EPIGENETIC MODIFICATIONS IN HUMANS

The research conducted on rats provides insight into the epigenetic mechanism of HPA axis dysregulation. Human research on this topic exhibits numerous similarities, and extends this work by exploring more diverse sources of early life adversity and linking long-term HPA axis perturbations to psychopathology. Most notably, the mechanism of methylation is highly conserved between rats and humans. Throughout numerous studies, children and adults who experienced early childhood abuse had increased methylation at the  $1_F$  region [34]. Since the  $1_F$  and the  $1_7$  homologs are highly conserved across species, the mechanism of GR regulation in both rats and humans likely operates through restricting transcription factor access to the promoter that drives GR expression [11]. Thus, although the prenatal and early life sources of stress may differ across species, they converge through a common mechanism to alter HPA axis activity. Interestingly though, contradictory studies have found both hypoactive and hyperactive HPA axis activity linked to childhood abuse [58-61]. However, for the studies presented here, discussion will be focused on the research that assesses aberrant HPA axis activity in the context of altered GR expression and early life stress, which predominantly reports hyperactive HPA axis activity.

##### 4.1. Prenatal Influences

Another area of similarity also lies within the fetal programming hypothesis as it applies to humans. As with rats, the human intrauterine environment is incredibly sensitive and influential in shaping developmental outcomes, thereby allowing fetal environmental programming to translate maternal experiences into stable phenotypic alterations *via* epigenetic control. However, since this defining period of developmental plasticity primes a fetal phenotype to be adapted to the intrauterine environment, the resulting phenotype may be maladaptive and at an increased risk for disease if the intrauterine environment is restricting or unreflective of the environment in which the newborn is raised [62]. Unsurprisingly, epigenetics in part mediates and contributes to the process of shaping the most adaptive phenotype [63].

A heavily studied prenatal influence is maternal mood and stress during pregnancy. In particular, maternal mood disorders during pregnancy have been associated with dysregulated neurodevelopmental outcomes [64]. Mood disor-

ders, especially depression, are characterized by hypercortisolemia and abnormal HPA axis activity [65, 66], and similar biochemical and neurobehavioral profiles have been found in infants of mothers with a mood disorder during pregnancy [67-69]. Normally, the fetus is protected against maternal cortisol since most of it is metabolized in the placenta, but abnormal elevations in maternal cortisol and the accompanying alterations in hormone and neurotransmitter circulation may modify the mother's metabolic and physiological processes, thereby allowing increased fetal exposure to cortisol [70, 71]. This altered intrauterine environment in turn influences the fetal neuroendocrine system and epigenetic profile. Indeed, increased levels of maternal cortisol during pregnancy have been directly associated with childhood affective problems [72].

Prenatal exposure to maternal depression, even when considered nonclinical, in the third trimester has been linked to increased methylation at the NGFI-A binding site in the  $1_F$  promoter region within mononuclear cells in the cord blood. Moreover, at three months old, newborns with increased *NR3C1* methylation also exhibited increased levels of salivary cortisol levels following exposure to a stressor, even when controlling for factors such as prenatal and postnatal maternal mood [12], demonstrating that methylation patterns independently inhibit HPA axis functionality at a very young age. Another study found promoter methylation to be associated with maternal cortisol levels and emotional state during pregnancy, particularly anxiety levels [73].

As a result of fetal HPA axis programming *via* glucocorticoid exposure and epigenetic control, the neurological and behavioral indices of stress in newborns have also been shown to be associated with *NR3C1* methylation. Infants whose mothers exhibited both higher indices of depression during pregnancy and greater placental methylation had abnormal behavior and exhibited depressive symptoms, which was reliant upon the presence of hypermethylation and thus also HPA axis activity [13]. Both gestational stress and subsequent alterations in newborn methylation in cord blood mononuclear cells have also been negatively correlated with newborn birth weight [74].

This work has been extended by broadly analyzing gestational stress that is not necessarily related to mood disorders. Radtke *et al.* conducted a retrospective analysis of adolescents whose mothers experienced intimate partner violence during pregnancy in order to determine if methylation patterns are sustained throughout development [75]. Using whole blood samples from children aged 10-19, increased methylation of the  $1_F$  region was only linked to intimate partner violence experienced during pregnancy, not before or after. Furthermore, methylation patterns in mothers' GR genes did not change as a result of intimate partner violence, demonstrating that epigenetic regulation of GR expression is not necessarily genetically inherited, but rather is a function of maternal stress during pregnancy. Another study found that both pregnant mothers exposed to the Tutsi genocide and their offspring exhibited hypermethylation within peripheral blood leukocytes as compared to mother-child pairs who were not exposed, suggesting that age is not necessarily an absolute restricting factor in *NR3C1* methylation, though age does still modulate sensitivity to stress exposure [10,

76]. The discrepancy between the mothers' methylation patterns in these two studies can potentially be resolved by considering the severity and chronicity of the stressors associated with the Tutsi genocide as modulators of the epigenetic response.

#### 4.2. Early Life Adversity and GR Expression

Similar to prenatal influences, childhood abuse and other sources of significant childhood stress are also associated with overactive HPA axis activity, increased risk for adult psychopathology, dysregulated hippocampal development, and increased risk for suicide - all of which are partially mediated by *NR3C1* methylation [77-81]. However, prenatal and early life experiences do not necessarily represent independent influences. Prenatal influences may carry over into early life experiences and alter child-rearing methods. Indeed, mothers with depression and anxiety have been shown to act less positively toward their children [82, 83].

There have been numerous studies that specifically analyze diverse sources of early life experiences that all converge onto a common outcome of *NR3C1* exon 1<sub>F</sub> promoter methylation at the NGFI-A binding site and enhanced HPA axis activity. One of the most commonly studied early life stressors is childhood abuse - including physical, emotional, and sexual abuse - which has been unequivocally associated with hypermethylation of the *NR3C1* promoter region [5, 84-86]. Instances of early parental death, neglect, and desertion have also been associated with hypermethylation, which has been shown to be directly related to altered cortisol levels [85, 87]. Another study continued to broaden the scope of early life adversities to encompass loss of a family member or friend, parental divorce, and trauma experienced during adolescence [84].

Due to the inaccessibility of the brain, the previously mentioned studies collected their genomic samples from saliva, whole blood analyses, and leukocytes. These changes may not necessarily correspond to similar epigenetic changes in specific brain regions, such as the hippocampus, a critical structure in maintaining the negative feedback loop within the HPA axis. However, McGowan *et al.* provided the first direct translation between rat studies and human studies by examining childhood abuse and GR methylation in the hippocampal regions [11]. Through postmortem genotype analysis, they found that suicide victims with a history of childhood abuse had significantly increased methylation of the *NR3C1* promoter in the hippocampus when compared to suicide victims with no history of childhood abuse and age-matched controls who had no history of childhood abuse and did not commit suicide. Thus, differences in GR expression are more closely correlated with familial dysfunctionality and adversity than with the actual act of suicide. However, given the involvement of GRs with the HPA stress response, stressors associated with decreased GR expression do appear to increase the risk of suicide [88, 89]. Moreover, as with the studies conducted on rats, this study also found decreased NGFI-A binding and GR mRNA transcripts in the hippocampal samples, thereby illustrating direct functional outcomes linked to *NR3C1* methylation that were still present in adulthood or at the time of death.

Aside from differential epigenetic profiles in hippocampal tissue, hypercortisolemia and mood disorders have also been robustly implicated in hippocampal atrophy [90-92]. Since GRs in the hippocampus regulate HPA activity, reduced hippocampal volume would impair their ability to contribute to the negative feedback loop. This could create a bidirectional cycle between epigenetic regulation of *NR3C1* and hippocampal volume that further exacerbates cortisol levels and has significant implications for psychopathology (Graphical Abstract).

#### 4.3. Psychopathology Vulnerability and Onset

Increased *NR3C1* promoter methylation derived from childhood adversity contributes significantly to the onset of psychopathology through developmental predispositions and HPA axis vulnerabilities [93]. After all, epigenetic modifications, specifically cytosine methylation, are known to be particularly stable, and *NR3C1* methylation stemming from childhood has been shown to last into adulthood [11, 36, 75]. These epigenetic alterations not only correspond to dysregulated HPA axis activity, but also to heightened physiological responses to stress - both of which are robust indicators for psychopathology vulnerability [94]. Thus, *NR3C1* promoter methylation may represent scars from childhood that persist into adulthood and mediate the onset of disorders associated with impaired stress responses.

A post-mortem analysis revealed decreased amounts of GR mRNA in brain tissue in patients with depression, bipolar disorder, and schizophrenia [95], similar to the results found in McGowan *et al.* [11] with suicide victims and childhood abuse that was linked to increased methylation and decreased mRNA expression. Increased *NR3C1* methylation in blood has also been observed in patients with borderline personality disorder [96], and a clear association between the development of borderline personality disorder and childhood adversity exists [97-99]. Moreover, another study found a positive correlation between the severity of childhood abuses, especially sexual abuse, and the degree of methylation of the *NR3C1* promoter in the peripheral blood in a sample of patients with borderline personality disorder [86]. A later study revealed two significant positive correlations also in a sample of patients with borderline personality disorder: one between childhood maltreatment and the degree of *NR3C1* methylation, and another between the degree of methylation and clinical severity. These trends were also observed in major depression, though to a lesser extent [86, 100]. Thus, in these conditions marked by adversity, there are both childhood maltreatment and *NR3C1* methylation underpinnings, suggesting that these methylation signatures represent a transdiagnostic risk factor for stress-related disorders.

However, definitive causality is difficult to establish. Even though childhood adversity has been associated with both epigenetic modifications and the development of psychopathology, and even though HPA axis activity is associated with both *NR3C1* promoter methylation and psychopathology, there must be caution in concluding causality. Future studies need to experimentally alter *NR3C1* expression to determine the variation in the stress response system, behavioral alterations, and the eventual development of psychopathology caused solely by *NR3C1*. This could be ac-

accomplished through genetic methods to experimentally modify GR expression levels, such as through gene knockout studies in rats. In addition to knockout studies, GR expression levels can be fine tuned through RNA silencing and promoter linked *NR3CI* construct experiments to better mimic how methylation alters gene expression. Methyltransferases, enzymes that methylate their targets, specifically guided to the *NR3CI* promoter could also accomplish the same goals. Moreover, broad epigenomic modification screens that indicate epigenetic changes across numerous genes would elucidate what other genes are affected by early adversity. Following these screens, the other genes can be controlled for in statistical analyses to isolate the effect of hypermethylated *NR3CI*.

#### 4.4. Potential Reversibility of Methylation Patterns

The contributions that early life adversity and *NR3CI* methylation make in the pathogenesis of mental disorders *via* increased predisposing vulnerabilities through altered HPA axis sensitivity, however, are indisputable. This poses the question of whether *NR3CI* methylation patterns can be reversed and whether this would correspond to phenotypic modifications in the stress system. In rat studies, it was demonstrated that *NR3CI* methylation patterns were reversible and corresponded to systemic and long-lasting changes in HPA axis functioning. Although McGowan *et al.* [11] provides the most direct translation of Weaver *et al.* [32] by demonstrating increased *NR3CI* methylation in human hippocampus following childhood adversity, it has still not been possible to thoroughly study reversibility and, secondary to that, causality in humans. Methylation patterns are certainly dynamic and respond to environmental factors and interventions, but this plasticity cannot be generalized to all genes [101, 102]. Research has suggested that psychotherapy can induce methylation changes in genes such as *BDNF* and *FKBP5*, but no changes were observed with *NR3CI* in patients with PTSD [103, 104]. However, this does not definitively mean that demethylation cannot occur in humans through other means or in a different sample of people. In fact, if anything, the results from rat studies should provide an optimistic outlook on treatment and intervention options for known victims of child abuse.

Evidence strongly suggests that the most sensitive period for acquiring stress-related methylation marks at the *NR3CI* promoter regions is before or during adolescence, though age is not necessarily an absolute restricting factor [10, 75]. This raises the question of whether the sensitivity window for reversing such marks, if possible at all, is similar. In rodents, the ability to alter phenotypic differences in stress reactivity was dependent upon the epigenetic state, not age [32]. However, this was accomplished through pharmacological agents that modify chromatin compaction state that cannot specifically target just *NR3CI* and thus cannot be applicable to humans. One method to test the sensitivity window for reversing the methylation patterns would be to identify child victims of abuse and adults with mood disorders. If they have increased methylation within *NR3CI*, then the first step would be to determine if various forms of psychotherapy decrease methylation. If a form of therapy is found that can produce robust effects, then determining how age modulates one's responsiveness to therapy-induced changes in methylation

patterns can be accomplished through exposing children, adolescents, and adults to therapy. Until advances in biomedical research uncover methods to directly alter the epigenetic state of specific genes, therapy remains the most viable option for potentially reversing the methylation marks.

#### 4.5. Stress through the Generations: The Epigenetic Transmission Cycle

It is feasible that *NR3CI* methylation linked to prenatal or early life experiences also propagates across generations *via* multiple interconnected mechanisms involving both nongenomic and genomic components. Thus, dysregulated stress response systems stemming from *NR3CI* alterations are not restricted to the individual who exhibits hypermethylation as a result of stress exposure, but could extend into future generations and also be a product of the stress experienced by one's ancestors. Although there is no human research on this topic, there is preliminary support for it from rodent research and indirect empirical support.

One potential mechanism for intergenerational transmission stems from the adoption of parental rearing styles. It has been observed that mothers and daughters exhibit similar behaviors towards their own children, thereby preserving maternal influences on child rearing throughout numerous generations [105, 106]. More broadly, child abuse is known to cycle through generations, such that parents abused as children are significantly more likely to abuse their own children [107-109]. Importantly, both genetics and environment significantly influence this and it is difficult to disentangle their individual contributions. It is likely that both operate in conjunction to transmit parenting styles. Studies analyzing either the developmental outcomes of twins reared apart or the children of twins elucidate the contributing factors, though decades of research indicate that both genetics and environment have strong influences on parenting styles [110]. Therefore, there are both genomic and nongenomic components involved in the transmission of parenting styles. Nonetheless, victims of such abuse demonstrate differential patterns of *NR3CI* methylation and are at increased risk for developing mood and substance abuse disorders [111]. Thus, cycles of abuse in itself represent one mechanism by which these methylation patterns can carry on through generations.

Another mechanism of intergenerational transmission operates through parental traumatization or other sources of parental stress. In fact, parental traumatization is not only associated with increased risk of their children developing mood disorders, but also with a higher incidence of abuse and child maltreatment, which are not independent events [112-114]. Indeed, another study found that mental health problems present in children were mediated by psychological maltreatment following parental trauma [115].

Aside from cycles of abuse, the later onset of psychopathology following either parental trauma or childhood maltreatment also poses the risk of propagating *NR3CI* methylation to the next generation. Drawing again from research on prenatal influences, neonates whose mothers had mood disorders not only demonstrated enhanced cortisol responses, but also depressive symptoms linked to hypermethylation [12, 13]. However, the effect of parental mood can extend beyond just the gestational period. Depressed and anxious

mothers have altered child-rearing behaviors, and generally react more negatively towards their children [82, 83]. Chronic stress and environmental adversity, which strongly characterize psychopathology, are known to produce variations in parental care and drive child abuse [52, 55, 116]. Thus, hypermethylation stemming from the intrauterine environment, which already represents compromised reactivity to stress and is stable enough to persist into adulthood, can interact with altered caregiving behaviors. This would in turn bolster the risk for their children to develop psychopathology and recreate a similar environment for their children, especially if these children later on have mood disorders during pregnancy [117]. However, it is once again likely that genetics and environment interact to transmit differential stress reactivities that may lead to psychopathology [118, 119].

Therefore, it is likely that HPA axis vulnerabilities mediated by *NR3CI* methylation contribute to and accompany the propagation cycles of psychopathology and child abuse. Indeed, research suggests that PTSD can be transmitted across generations through its conferred epigenetic risk and negative influence on parenting. Interestingly, however, GR expression increases in those with PTSD, and the results discussed are not inclusive of this disorder [10, 120-122], (for an in depth review of PTSD [113]).

#### 4.6. The Effects of Gene-Environment Interactions on GR Expression

The joint impact of genetic predispositions and environmental stressors must be considered when analyzing group differences in *NR3CI* methylation. Other genes have been implicated in regulating GR function and modulating individual sensitivity to stressors, in particular *FKBP5* [19]. Most notably, single nucleotide polymorphisms (SNPs) in the human *FKBP5* gene have been shown to regulate the HPA axis and differentially predispose people to psychological disorders [123]. This gene encodes for FK506 binding protein 5 (FKBP5), which binds to GRs and decreases their affinity for cortisol, thereby serving as an inhibitor of GR functionality. Moreover, activation of GRs induces FKBP5 expression, which then creates a short negative feedback loop for GR function [124].

Recent research has shown that three different *FKBP5* gene SNPs - rs4713916, rs1360780, and rs3800373 - were associated with the prevalence and severity of depressive periods [125]. More specifically, the rs1360780 T allele has been shown to lead to greater FKBP5 induction following GR activation, thereby leading to poor HPA axis regulation. In healthy individuals with the rs1360780 T allele, prolonged cortisol responses are observed following psychosocial stress [126]. In individuals with this SNP allele who experienced childhood abuse, there was significant demethylation in the *FKBP5* gene, which increased FKBP5 expression and thus GR resistance [127, 128]. However, in rs1360780 C allele individuals who experienced similar childhood trauma, no demethylation of the *FKBP5* gene was observed [129]. Thus, genetic variations within the *FKBP5* gene can alter epigenetic regulation of that gene, which can then predispose individuals to psychiatric disorders through FKBP5 regulation of GRs. Even though this gene-environment interaction does not alter the epigenetic regulation of *NR3CI*, it does regulate

epigenetic changes in another gene that alters GR functionality. Further studies are needed to confirm if methylation patterns of *NR3CI* are mediated by the genetic variations in the *FKBP5* gene.

Furthermore, there has been preliminary research conducted on SNPs within *NR3CI*, and initial results suggest that there are gene-environment interactions mediating highly stressful life events and the subsequent risk of developing PTSD [130]. However, the authors of the study note that many of the associations found have yet to be replicated, and meta-analyses compiling future replications are needed before conclusive results can be drawn. They also note that SNP frequencies in the population are low, thus creating a limited impact for a single SNP on the progression of HPA axis development [131]. These interactions, however, are still important to uncover and may provide insight into new treatments and clinical outcomes.

#### CONCLUSION AND FUTURE DIRECTIONS

A major challenge in studying epigenetic regulation of the stress response is the inaccessibility of the human brain, especially during childhood in the midst of adverse experiences. To date, no studies have been able to actively analyze GR expression and *NR3CI* methylation in brain tissue during the most sensitive time periods of neurodevelopment. The closest findings stem from post-mortem hippocampal analyses, and many human studies rely on measurements from peripheral blood, saliva, umbilical cord, immune cells, and placental cells. Even though epigenetic patterns tend to differ between cell types and organ systems, there is considerable evidence that peripheral measurements can translate well to brain measurements [132].

From the extensive studies conducted on rats, adverse experiences were associated with increased methylation at the exon 1<sub>7</sub> NGFI-A binding sequence in both the periphery and brain, and these findings have been independently reported at the human homolog, exon 1<sub>F</sub>, from numerous human studies spanning multiple tissue types, suggesting conservation of mechanism between the two species. Moreover, it has been shown that individual differences in *NR3CI* methylation patterns in saliva are associated with memory related cognitive processes, suggesting a functional link between peripheral biology and the hippocampus [33]. Recent studies are increasingly finding more associations between methylation signatures in the periphery and the brain, especially as it pertains to *NR3CI*, thereby encouraging the assumption of similar epigenetic patterns for future studies [133-136]. However, caution must still be exerted in these correlative studies until definitive evidence uncovers associations between peripheral and central nervous system methylation patterns.

Yet despite the accumulating evidence, definitive proof of a connection between peripheral and brain measurements derived from the same sample of participants is needed. Even if differences in methylation patterns are found, the findings would provide novel insight into the developmental mechanisms of differential *NR3CI* methylation and psychiatric pathogenesis, though it is unlikely significant differences in methylation patterns between body regions would exist [137]. However, it still remains to be shown that increased

methylation is associated with both decreased expression of mRNA transcripts bearing the 1<sub>F</sub> splice variant and decreased GR expression in all tissue types. This research is necessary to establish a comprehensive functional link between epigenetic processes, cellular processes, physiological mechanisms, and ultimately, behavioral outcomes. A related area of future investigation would be to uncover the signaling and molecular pathway that links hypercortisolemia to epigenetic changes within *NR3C1*, and if this pathway differs by tissue region. This could address the compensatory function of GR downregulation and the degree of conservation in molecular processes subserving the epigenetic processes that modulate stress reactivity. Knowledge of such a pathway would also have implications for reversing the methylation patterns through pharmacological interventions.

The degree of variation in physiological, behavioral, and developmental outcomes due to altering expression of solely *NR3C1* also needs to be studied so that its individual role in altering the stress response system and predisposing individuals to psychopathology can be better understood. It has been reported that childhood maltreatment also decreases methylation within *FKBP5*, thereby increasing GR resistance and amplifying the stress response even further [138]. There are likely numerous other factors, both genetic and environmental, confounding the effects of *NR3C1* methylation. Thus, studying effects from altering just *NR3C1* would clarify its individual role in mediating the stress response.

Moreover, many studies specifically analyzed CpG segments within the promoter region, and specifically the NGFI-A binding site within exon 1<sub>F</sub>. While this provides informative data, future research should examine whether these are the only specific sites where methylation occurs, if this is modulated by regional specificity within the body, and if methylation imprints occur elsewhere, whether those are associated with differential functional outcomes. Given that there are seven different alternative splicing sites within the first exon, the other promoter regions would be informative candidates for study.

Another limitation in studying early life adversity is that causality is difficult to establish due to the infeasibility of manipulating experimental control in human studies. It is also difficult to disambiguate the coincident circumstances that may accompany early life adversity. However, the studies conducted on rats provide insight into disentangling potential confounding variables. Since the mechanism of epigenetic regulation is highly conserved across rats and humans, the data obtained from human studies closely parallel and extend the data obtained from rat studies. Moreover, since the rats were cross-fostered by different parents and raised in similar external environments, it is possible to address confounding variables such as genetic predispositions, nutrition, and general external environment.

Although these studies may not translate perfectly to humans, the various studies that did analyze differential GR expression controlled for factors such as changes in maternal GR expression, magnitude of early life adversity, and sociodemographic characteristics of the parents, such as education, income, and marital status. However, further research, especially twin studies, is needed to elucidate the extent to which the environment modulates GR expression in humans.

It is also worthwhile to study prenatal and childhood stressors together to determine the nature of their interactions on methylation status.

Although the HPA axis can certainly be adaptive, stress induced alterations in HPA axis regulatory mechanisms can cause excessive overreaction of the stress response. While this may be evolutionarily beneficial and adaptive in incredibly adverse environments, it is maladaptive in the vast majority of situations and leads to detrimental stress responses [139, 140]. The findings presented in this review provide insight into the precise mechanisms by which early childhood maltreatment can predispose psychopathology and negatively influence developmental plasticity through dysfunctional epigenetic programming. It is also highly plausible that these epigenetic modifications can then propagate themselves *via* the creation of anxiety and mood disorders in adults abused as children, who then create similarly stressful environments for their children.

Ultimately, these studies can serve as the foundation for understanding interventions that can potentially reverse *NR3C1* methylation and predispositional HPA axis vulnerabilities. As such, the findings presented have the potential to be translated into better prevention programs and treatment options for victims of abuse and various other early childhood traumas, provided that future research fully elucidates the extent to which genetic and environmental factors influence GR expression and how intervening factors can modulate each one.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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