Early life trauma, depression and the glucocorticoid receptor gene – an epigenetic perspective

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Abstract

Hopes to identify genetic susceptibility loci accounting for the heritability seen in unipolar depression have not been fully realized. Family history remains the gold standard for both risk stratification and prognosis in complex phenotypes such as depression. Meanwhile, the physiological mechanisms underlying life-event triggers for depression remain opaque. Epigenetics, comprising heritable changes in gene expression other than alterations of the nucleotide sequence, may offer a way to deepen our understanding of the aetiology and pathophysiology of unipolar depression and optimise treatments. A heuristic target for exploring the relevance of epigenetic changes in unipolar depression is the hypothalamic-pituitary-adrenal (HPA) axis. The glucocorticoid receptor (GR) gene (*NR3C1*) has been found to be susceptible to epigenetic modification, specifically DNA methylation, in the context of environmental stress such as early life trauma, which is an established risk for depression later in life. In this review we discuss the progress that has been made by studies that have investigated the relationship between depression, early trauma, the HPA axis and the *NR3C1* gene. Difficulties with the design of these studies are also explored. Future efforts will need to comprehensively address epigenetic natural histories at the population, tissue, cell and gene level. The complex interactions between the epigenome, genome and environment, as well as ongoing nosological difficulties, also pose significant challenges. The work that has been done so far is nevertheless encouraging and suggests potential mechanistic and biomarker roles for differential DNA methylation patterns in *NR3C1* as well as novel therapeutic targets.

Introduction

The scale of present day psychiatric illness is well documented. It is thought to account for nearly 23% of the disease burden in the UK (Fineberg *et al.*, 2013) and is similarly prominent in worldwide estimates (Murray *et al.*, 2012). One of the largest diagnostic contributors to this burden is unipolar depression, which has a global lifetime prevalence rate of up to 17% (Flint and Kendler, 2014). Unipolar depression is projected to become the greatest worldwide cause of disability-adjusted life years (DALYs) by the year 2030 (WHO, 2008) and is already associated with significant mortality (Antypa *et al.*, 2013). Thus, there remains an ever-growing need to further our understanding of depression in order to optimise treatments and to alleviate suffering (Collins *et al.*, 2011). Deterministic genetic models for psychiatric illness have proved elusive. Genome-wide association studies (GWAS) have not translated into significant therapeutic gains for disorders such as depression and aetiology from a genetic viewpoint remains opaque (Lewis *et al.*, 2010; Ripke *et al.*, 2013). Possible reasons for this include well-cited nosological difficulties (Casey *et al.*, 2013) incorporating phenomenological, psychopathological and pathophysiological heterogeneity, small effect sizes of individual genes and overestimated heritability (McGuffin *et al.*, 2007; Bohacek and Mansuy, 2013; Uher, 2014). However, a growing body of evidence suggests that epigenetic modification could be a biologically significant factor in depression with potential diagnostic, prognostic and therapeutic uses (Massart *et al.,* 2012; Bohacek *et al.*, 2013; Sun et al., 2013; Bagot *et al.,* 2014).

Heritability of depression

For many years twin and family studies have highlighted the importance of genetic and environmental factors in the mediation of the vulnerability to depression (Cohen-Woods *et al.*, 2013). In the first meta-analysis of epidemiological studies in depression, Sullivan *et al.* (2000) compared susceptibility in monozygotic and dizygotic twins. The majority of the variance in liability was attributed to environmental effects specific to the individual (63%), whilst genetic effects accounted for 37%. Subsequent studies have

produced similar results (Kendler *et al.*, 2006; Franz *et al.*, 2011; Nivard *et al.*, 2014). This genetic contribution has been examined using linkage and association studies. Gene linkage studies for depression, as well as for other common complex disorders, have been perceived by some to be of only limited success (McGuffin *et al.*, 2007; Nair and Howard, 2013) hence the focus on GWAS. However, even for simple traits, genetic variants identified by GWAS are rarely shown to account for more than 20% of the heritability (Wood *et al.,* 2014). GWAS has had limited success for depression in finding significant associations with individual genetic variations and there has been no evidence for a recessive model (Cohen-Woods *et al.*, 2013; Chang *et al.*, 2014; Flint and Kendler, 2014; Levinson *et al.*, 2014; Power *et al.*, 2014; Schneider *et al.*, 2014). Even when technical and statistical aspects of GWAS have been taken into account (Gusev *et al.*, 2013) the extent of this disparity between expected and verified genetic components in depression has remained considerable (Castillo-Fernandez *et al.*, 2014) and family history continues to be the most effective method of predicting risk (Maher, 2008). Nevertheless, trying to dovetail epidemiological studies which suggest a considerable genetic component for depression with genetic studies providing a dearth of single nucleotide polymorphism (SNP) associations remains a significant problem.

The HPA axis

One major endocrinological finding in depression is of a dysregulation of the HPA axis – a pathway important in regulating stress responsivity. Stress has profound effects upon a broad range of physiological systems and is an established trigger for mental illness (Meaney, 2001; Gallagher *et al.*, 2007; Binder *et al.,* 2008; Turner *et al.*, 2010; Klengel *et al.*, 2014). The HPA axis is the foremost neuroendocrine stress response system. Dysregulation of the HPA axis in unipolar depression has been consistently reported since the 1960s (Gibbons, 1964; O'Toole *et al.*, 1997; McAllister-Williams *et al.*, 1998; Holsboer, 2000; Young, 2004; Moser *et al.*, 2007; Pariante and Lightman, 2008). Neuroendocrine studies have shown increased basal and/or activated levels of the HPA axis hormones – corticotropin-releasing hormone (CRH), vasopressin (AVP), adrenocorticotropic hormone (ACTH) and cortisol – in

plasma, saliva and cerebrospinal fluid (CSF) (Herbert, 2013; Belvederi Murri *et al.*, 2014). Structural changes have also been seen in post-mortem and imaging studies of depressed patients including increased numbers of CRH secreting neurones in the hypothalamus (Raadsheer *et al.*, 1994) and enlarged pituitary and adrenal gland volumes (Kessing *et al.*, 2011). Meanwhile non-suppression in the dexamethasone/ CRH test has been associated with inferior treatment response and increased relapse rates in depression (Aubry *et al.*, 2007; Ising *et al.*, 2007; Medina *et al.*, 2013).

The HPA axis is regulated by negative feedback loops incorporating glucocorticoids (GC) and glucocorticoid receptors (GR) (Alt *et al.*, 2010). Thus, studies exploring the mechanism underlying the HPA axis dysregulation that has been documented in depression have focused upon abnormal GR expression and function (Pariante, 2006 and 2009; Cowen, 2009; Anacker *et al.*, 2011). Indeed preclinical and clinical investigations have implicated a significant role for GR abnormalities in depression. Using knock-out mice Boyle *et al.* (2005) demonstrated that reduced GR function led to disrupted negative feedback inhibition of the HPA axis and depression-like behaviour. Depressed patients have shown reduced peripheral GR levels and increased 24-hour cortisol levels (Yehuda *et al.*, 1993). In line with this, decreased GR mRNA has been demonstrated in post-mortem frontal cortices from depressed patients (Webster *et al.*, 2002), although a more recent study has found increased GR expression in amygdala samples from depressed patients (Wang *et al.*, 2014). These findings have been bolstered by studies implicating improved GR function and increased GR expression in the mechanism of action of certain antidepressants (Pariante and Miller, 2001).

Early life adversity, depression and the HPA axis

The impact of maternal care and early life adversity has been investigated across several species. Rodents temporarily separated from their mothers in the first few months of life, and those whose mothers provide low levels of licking and grooming (LG) and arched back nursing (ABN) care (Meaney, 2001; Daskalakis *et al.*, 2013), have been shown to exhibit depression-like

phenotypes (Caldji *et al.*, 2000; Murgatroyd *et al.*, 2009). Early adversity has also been associated with abnormal HPA axis function (Fish *et al.*, 2004; Archer *et al.*, 2014). Liu *et al.* (1997) demonstrated that high LG-ABN maternal conditions, triggered by brief human handling, were associated with lower levels of plasma adrenocorticotrophic hormone (ACTH) and corticosterone (CS) in response to stress in rat offspring up to 100 days old. These offspring also had greater GC sensitivity, increased levels of hippocampal GR mRNA and lower levels of hypothalamic CRH mRNA. In an associated study, Francis *et al.* (1999) showed that rat pups cross-fostered from low to high LG-ABN mothers had dampened behavioural responses to stress. More interesting still, these offspring emulated their high LG-ABN foster mothers when caring for their own offspring, while low LG-ABN foster mothers rearing pups from high LG-ABN biological mothers produced offspring with heightened stress responses and low LG-ABN maternal behaviour. Further studies by Murgatroyd and Nephew (2013, and another study currently in submission) have been able to show that exposing rat mothers to chronic stress during lactation leads to reduced levels of maternal care as well as altered neuropeptide regulation and GR expression. Moreover, pups whose mothers were exposed to chronic stress tend themselves to exhibit reduced maternal care in adulthood. These results supported prior studies (Denenberg and Rosenberg, 1967; Danchin *et al.*, 2011) demonstrating that differences in gene expression could be passed from one generation to the next by non-genomic means. Further work has been carried out on epigenetic transgenerational inheritance and it continues to attract much attention (Bohacek *et al.*, 2013; Crean *et al.*, 2014; Babenko *et al.*, 2015).

In humans early life adversity is acknowledged as a significant risk factor for many psychiatric and non-psychiatric illnesses (Rutter, 1985; Meaney, 2001; Maniglio, 2009). Childhood maltreatment – incorporating physical, emotional and sexual abuse and physical and emotional neglect – is a significant source of early life adversity in human populations. In Britain, childhood maltreatment has been estimated to occur in 15-25% of the population (May-Chahal and Cawson, 2005; Radford *et al.*, 2013) with comparable figures quoted internationally (Ishida *et al.*, 2013; Barbosa *et al.*, 2014; Finkelhor *et al.*, 2014;

Wildeman *et al.*, 2014). Those who are subjected to childhood maltreatment are thought to be at greater risk of later life depression (Klengel *et al.*, 2014). More specifically, Bifulco *et al.* (1991) demonstrated an increased risk of depression in women who had been abused as children and other subsequent studies have supported these findings (Kendler *et al.*, 2000; Widom *et al.*, 2007; Alt *et al.*, 2010; Bale *et al.*, 2010; Heim et al., 2010). Such individuals can exhibit persistent neuroendocrine and anatomical changes including: glucocorticoid insensitivity, increased central CRH activity, immune upregulation and reduced hippocampal volume (Heim and Nemeroff, 2001; Heim *et al.*, 2008; Hornung and Heim, 2014). Similar abnormalities have been seen in parentally bereaved children in the form of elevated 24-hour salivary cortisol concentrations (Nicolson, 2004) and elevated cortisol in the dexamethasone/ CRH test (Tyrka *et al.*, 2008). However, stress diathesis remains a complex phenomenon with no absolute demarcation between brief (potentially beneficial) and persistent (potentially damaging) stress. In some instances early adversity has appeared to prime, or 'stress inoculate', the individual to later adversity (Anisman *et al.*, 1998; Carpenter *et al.*, 2007; Watson *et al.*, 2007; Elzinga *et al.,* 2008; Daskalakis *et al.*, 2013). Thus, a relationship clearly exists between early adversity, depression and HPA axis function. The potential for individual genes to exert a mediating role in this relationship is the subject of much current study. One such gene is *NR3C1*.

The NR3C1 gene

NR3C1 is found on chromosome 5q31-32 and is over 150kb in length (Franke and Foellmer, 1989; Turner *et al.*, 2014). It has 8 translated exons (numbered 2 to 9) and is thought to have up to fourteen untranslated alternative first exons (termed 1a through to 1j, with 1a and 1c having six further sub-divisions between them) at its 5' end (Daskalakis and Yehuda, 2014). *NR3C1* has a complex promoter structure with one promoter for each of its single alternative first exons (Turner *et al.*, 2010). Alternative first exon transcripts are thought to be important for adjusting GR levels in accordance with cell or tissue type and dynamic environmental conditions (Turner *et al.*, 2006) by differentially regulating translation efficiency and RNA stability (Bockmühl *et al.,* 2011). Transcription factors known to modulate alternative first exon use include

nerve growth factor inducible protein A (NGFIA) which binds to the exon 1f promoter (Weaver *et al.,* 2004). GR is a ligand-activated transcription factor crucial for the effective functioning of the HPA axis. It translocates to the nucleus after binding GC to regulate the activity of specific target genes, including *NR3C1* itself. *NR3C1* splice variants and mRNA levels, GR isoforms, co-activators and co-repressors have all been associated with variations in GR activity (Binder, 2009; Turner *et al.*, 2010; Anacker *et al.*, 2011; Szczepankiewicz *et al.,* 2014). Although GR is ubiquitously expressed, its levels are thought to be tightly controlled according to tissue, or even cell, type. Thus, within the brain, levels are higher in areas involved in the stress response such as the paraventricular nucleus, hippocampus and anterior pituitary (Karanth *et al.*, 1997; Uchida *et al.*, 2008; Booij *et al.*, 2013).

Epigenetics

Epigenetic mechanisms - encompassing DNA methylation, histone modification and non-coding RNAs - act to modulate gene expression (Bird, 2002; Bird, 2007; Weaver *et al.*, 2007; Akbarian and Huang, 2009; Bale *et al.*, 2010; Murgatroyd and Spengler, 2011; Booij *et al,*, 2013) and arguably have the potential to explain the extent and nature of the risk of depression conferred by the interaction between environmental and genetic factors. Factors determining the extent of the epigenetic modifications include cell type, developmental stage and the nature and severity of environmental stressors.

DNA methylation, the most widely studied epigenetic modification, involves the addition of a methyl group to the 5-carbon position of a cytosine at the 5' end of cytosine-guanine dinucleotides (CpG sites). CpG clusters ('islands') are often present within gene promoter regions in an unmethylated state but when they become methylated the gene with which they are associated is usually silenced. Methylation is thought to exert this silencing effect, in concert with other epigenetic modifications and signalling pathways (Day and Sweatt, 2011; Reul, 2014), by inhibiting the binding of transcription factors to promoter regions. Gene promoter regions, as well as the main gene bodies themselves, have commonly been investigated for differential methylation patterns but more recently methylation within enhancer elements has also been

recognised (Plank and Dean, 2014). The impact of the location and the extent of methylation required to prevent gene expression remains unclear but, in cancer studies using discordant monozygotic twins, large effects have been seen with absolute increases in methylation of 10% or less (Galetzka *et al.*, 2012; Heyn *et al.*, 2013). DNA methylation, acknowledged as an important factor in tissue development for many years (Holliday and Pugh, 1975; Riggs, 1975; Razin and Riggs 1980), has emerged as a potential underlying mechanism for changes in GR expression.

Figure 1:
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Early life adversity and methylation of *NR3C1* in animals

In 2004 Weaver *et al.* demonstrated that in rat pups of low LG-ABN mothers *NR3C1* methylation was increased in hippocampal samples (to rates of 80-100%) within the NGFIA binding site of the GR gene's exon 1(7) promoter, the homologue of exon 1f in humans, and that this was associated with reduced GR expression. Conversely they showed that high LG-ABN maternal care was associated with lower methylation rates (0-10%) of the exon 1(7) GR promoter in offspring. In agreement with a prior study (Meaney *et al.*, 1996) examining GR expression these differences in methylation rates persisted into the offspring's adulthood. Group differences were eliminated by trichostatin A, a histone deacetylase inhibitor thought to promote demethylation (Ou *et al.*, 2007), as well as by cross-fostering. This was the first study in the literature to show a clear link between mothering, long-term changes in DNA methylation patterns and subsequent gene expression. Further studies were conducted with, at times, conflicting results. Daniels *et al.* (2009), for instance, investigated the impact of separating rat pups from their mothers between postnatal day 2 and 14. Assessment of hippocampi at postnatal day 21 revealed no significant change in methylation within exon 1(7) or the NGFIA binding site between pups separated from their mothers and pups raised normally. Nevertheless, Weaver *et al.* (2007) demonstrated that high LG-ABN care was associated with demethylation of the 5' CpG dinucleotide in the NGFIA response element specifically. Additionally, the authors showed that increased LG-ABN was correlated with greater NGFIA binding, histone acetylation, GR mRNA levels, hippocampal NGFIA expression and increased amounts of GR protein.

Early life adversity and methylation of *NR3C1* in humans

One of the first studies in humans to examine the relationship between prenatal adversity and *NR3C1* methylation was by Oberlander *et al.* (2008). Children of depressed mothers who had received medication (n=33) were compared with children of untreated depressed mothers (n=13) and controls (n=36). An association was seen between prenatal exposure to third trimester

maternal depression and increased methylation levels of the NGFIA binding site in exon 1f of the *NR3C1* promoter (deemed CpG sites 1 to 3) at birth. CpG site 3, within the NGFIA binding site, was also associated with increased cortisol response at 3 months of age. Antidepressant medication had been associated with increased GR mRNA levels in rodents (Pepin *et al.*, 1992; Pariante and Miller, 2001; Yau *et al.*, 2002) and increased GR density in human peripheral blood cells (Calfa *et al.*, 2002), whilst *NR3C1* polymorphisms have been observed to predict antidepressant medication responses (Binder *et al.*, 2004; Spijker and van Rossum, 2012). However, maternal treatment with selective serotonin reuptake inhibitors (SSRIs) in the Oberlander *et al.* study did not have any observable effect on offspring CpG methylation status. Nevertheless, these findings encouraged other studies (Hompes *et al.* 2013 for example) to assess the same portion of the exon 1f promoter and CpG sites. Conradt *et al.* (2013) reported that newborn offspring exposed to maternal depression *in utero* had increased methylation at the authors' CpG site 2 within exon 1f as well as adverse neurobehavioural outcomes.

Radtke *et al.* (2011) examined DNA methylation using peripheral blood samples taken from children (n=24) aged up to 19 years old whose mothers had been exposed to violence before, during and after their pregnancy. Increased methylation rates in children were significantly associated with maternal exposure to violence during their pregnancy. Methylation was seen in 7 of the 24 children, in 5 of the 10 CpG sites examined and at rates of up to 10%. Strikingly, there was no association between child *NR3C1* methylation and maternal exposure to violence either before or after pregnancy. Maternal *NR3C1* methylation was not significantly correlated with methylation levels in their children and was unaffected by exposure to violence. This study was the first in humans to show an apparently sustained dysregulation of the HPA axis associated with previous early life psychological stress. However, the lack of data over such a long periods of time, up to 19 years in some instances, and the small sample sizes used meant that innumerable confounders could not be ruled out and the statistical power of the study remained relatively limited.

Such site-specific findings, again using the portion of the exon 1f adopted by Oberlander *et al.* (2008), were seen in a study by Tyrka *et al.* (2012) in which

99 healthy adult subjects showed correlations between *NR3C1* methylation at CpG sites 1 and 3 in the exon 1f promoter and previous childhood maltreatment, parental care and parental loss. However, methylation rates at these sites and cortisol response were not correlated and the study did not incorporate gene expression data. Hence, the functional significance of their findings remained debatable.

Depression and methylation of *NR3C1*

In a study using buccal DNA from healthy individuals (n=92) Edelman *et al.* (2012) were able to show that methylation at a single CpG site within a binding site for NGFIA correlated with cortisol response to stress. Furthermore, as a result of epigenetic modifications being chemically stable yet modifiable in accordance with dynamic environmental factors (Meaney and Szyf, 2005; Sweatt, 2009) *NR3C1* methylation has been afforded considerable explanatory potential in trying to understand both HPA axis dysregulation and depression (Turner *et al.*, 2010). Inconsistent results from *NR3C1* SNP studies in depression (Bouma *et al.,* 2011; Lahti *et al*., 2011; Lewis *et al.,* 2011; Engineer *et al.,* 2013; Galecka *et al.,* 2013; Koper *et al*., 2014) have added further impetus to this field of enquiry.

In 2010 Alt *et al.* conducted a study exploring the possible association between methylation of *NR3C1* and depression. The authors assessed *NR3C1* methylation in post-mortem samples from depressed patients (n=6) in multiple limbic brain regions compared to controls (n=6). Hippocampal exon 1f transcripts were reduced in depressed patients and NGFIA was down-regulated within the hippocampus, cingulate gyrus and nucleus accumbens. However, these data demonstrated very low overall levels of methylation in both depressed and control brains, whilst the *NR3C1* promoter for exon 1f was completely unmethylated in all of the samples taken. Thus, the mechanism for this down-regulation in depressed brains appeared to be entirely independent of methylation patterns. However, as the authors themselves acknowledged, this study's power was limited by small sample sizes. More recently Na *et al.* (2014) compared methylation levels of *NR3C1*'s promoter region in depressed patients (n=45) and controls (n=72). The authors found hypomethylation, rather than hypermethylation, at two CpG

sites in patients. Neither the Alt *et al.* (2010) nor the Na *et al.* (2014) studies were able to provide definitive data regarding HPA axis functioning or childhood trauma. This would appear to be crucial, as illustrated by the following studies incorporating early adversity into models of depression, HPA axis dysfunction and methylation of *NR3C1* at different life stages.

Early life adversity, depression and methylation of *NR3C1*

McGowan *et al.* (2009) compared methylation rates in hippocampi of post-mortem samples from suicide victims, with and without histories of childhood abuse, and controls (n=12 for each of these three groups). Two thirds of the suicide victims, whether abused or not, were retrospectively diagnosed with mood disorders via psychological autopsies (DSM-III-R). Levels of childhood abuse in suicide victims correlated with higher levels of methylation of the *NR3C1* promoter as well as lower *NR3C1* mRNA levels, both overall and for the exon 1f splice variant alone. There was no significant difference in methylation rates of the exon 1f *NR3C1* promoter or *NR3C1* mRNA levels between non-abused suicide victims and controls. In abused suicide victims CpG site-specific increases in methylation were associated with reduced NGFIA binding and NGFIA-induced transcription. However, this study was limited statistically by the small sample sizes used for each group and the removal of outliers in their final analysis. Also of note was that McGowan *et al.* (2009) reported exon 1f levels accounting for up to 60% of the total amount of expressed *NR3C1* promoters. Alt *et al.* (2010), meanwhile, gave a figure in accordance with previous studies of less than 1%. Such relatively low levels of exon 1f could detract from its apparent functional importance in comparison to other alternative first exons. Whilst dramatically different expression rates between studies may also reduce the confidence with which results can be generalized.

Perroud *et al.* (2011) investigated the correlation of the severity of childhood maltreatment with methylation rates in *NR3C1* for patients diagnosed with borderline personality disorder (BPD) (n=101), depression (n=99) or depression with co-morbid post-traumatic stress disorder (n=15). A portion of the exon 1f promoter was analysed with reference to the sequence used by

Oberlander *et al.* (2008). They were able to show highly significant associations between methylation of *NR3C1* and the severity and number of sexual abuse episodes. In a study by Melas *et al.* (2013) salivary DNA from depressed adults (n=92) were compared with controls (n=82). They examined whether differential methylation rates of *NR3C1* were associated with various adversities experienced in childhood. Of the 47 CpG sites spanning the exon 1f promoter that were analysed increased methylation was seen at a single CpG site, near to the NGFIA binding region. This was significantly associated with early parental death. However, this study did not assess childhood abuse, the numbers involved in the adversity sub-groups were not large and only females were included.

Very few studies have identified candidates for human maternal behaviours equivalent to rat LG-ABN. However, in rats the effects of licking and grooming have been shown to be mimicked by stroking pups with a brush (Mulligan *et al.,* 2012). A study by Sharp *et al.* (2012) demonstrated moderation of the effects of prenatal maternal depression upon emotional and physiological outcomes in human infants through mothers stroking their babies in their first weeks of life. A very recent follow-up study by Murgatroyd *et al.* (2015) has showed reduced *NR3C1* methylation associated with maternal stroking in these children, hence bolstering the possible role of epigenetic mechanisms in the long-term effects of early life stress and maternal care. Interestingly, the same study also found interactive effects between prenatal and postnatal maternal depression on methylation of *NR3C1*'s exon 1f. Infants of mothers with low prenatal depression showed increased methylation when exposed to increased postnatal depression - consistent with an interplay between prenatal and postnatal environments. In general terms this is supportive of the foetal origins hypothesis of human disease according to which environmental exposures *in utero* lead to adaptive modifications in foetal development that act to increase fitness in similar postnatal environments.

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Methodological issues and possible future studies

Whilst these human studies have added overall support to a role for epigenetic modification in the link between early adversity, HPA axis dysregulation and depression vulnerability, attention is increasingly being drawn to inconsistencies in study design that may have prevented causal inferences being made (Daskalakis and Yehuda, 2014; Turecki and Meaney, 2014). In this final section an attempt will be made to give an overview of such inconsistencies in order to offer a potential direction for future studies.

Unlike the majority of animal studies examining *NR3C1* methylation, many types of potential stressors, sometimes at different developmental stages, have been used to represent early human adversity. For example, Oberlander *et al.* (2008) used prenatal exposure to maternal depression whilst Radtke *et al.* (2011) examined the impact of pre- and perinatal exposure to violence against the mother. Both McGowan *et al.* (2009) and Perroud *et al.* (2011) used histories of childhood abuse. Studies examining the impact of early adversity in humans is clearly more limited in design by ethical considerations when compared to animal studies. Given the relatively complex nature of human interactions and stress diathesis there is a need to minimise confounders by standardising the assessment of stressors whenever possible. With regards to using stressors at different developmental stages, the natural history of site-specific methylation, such as the exon 1f promoter of *NR3C1*, in individual subjects has not been explored. Prenatal stress exposure, as used by Oberlander *et al.* (2008) and Radtke *et al.* (2011), has helped to establish the temporal boundaries of what appears to be a developmentally sensitive period for a possible causal chain of adversity, epigenetic modification, HPA dysregulation and subsequent depression. However, substantial differences can be expected in the nature of stresses prenatally compared to postnatally, as well as their developmental consequences, and this again risks introducing many confounders when attempting to interpret data. Future studies need to comprehensively detail adverse events, as is common practice on psychiatric inpatient wards and to a

lesser degree in the community setting, over extensive periods of time and to combine this with regular assessments of epigenetic modifications.

Human studies have also undertaken analysis in different types of tissue. Peripheral blood has often been used due to its relative acceptability from the patient's perspective and its clinical practicality. Radtke *et al.* (2011) and Perroud *et al.* (2011) used DNA extracted from peripheral whole blood whilst Oberlander *et al.* (2008) investigated mononuclear cells from the cord blood of newborns. Other studies have looked at post-mortem tissue: McGowan *et al.* (2009) analysed hippocampal specimens whilst Alt *et al.* (2010) looked at several different limbic regions. Melas *et al.* (2013), meanwhile, used DNA from saliva. It has already been highlighted that methylation levels may differ between cell types (Glossop *et al.,* 2013; Simar *et al.,* 2014) meaning that comparisons between studies using entirely different tissues could be very challenging. However, evidence has emerged that peripheral blood may be an appropriate tissue to identify biomarkers for depression in the context of genetic studies (Rollins *et al.* 2010; Hepgul *et al.,* 2013), whilst in a review by Tylee *et al.* (2013) the methylome was shown to be more highly correlated between blood and brain samples than the transcriptome. Provencal *et al.* (2012) showed that in rhesus macaques variations in mothering (surrogate versus mother reared) led to differential methylation rates including the *A2D681* gene which is the homologue of *NR3C1* in humans. A weak but significant correlation was seen in differential methylation between prefrontal cortex samples and T lymphocyte cells. The use of blood samples does have an advantage over the use of post-mortem and placental tissue given that these samples are taken at markedly different physiological states. Saliva samples involve cells from different developmental lineages to both brain and blood tissue and hence the validity of its use is unclear. These various confounders mean that many more studies will be needed before effects directly attributable to early life trauma can be separated from those relating to tissue type. Future investigations also need to involve repeated peripheral samples taken from individuals who have nominated themselves for future brain donation. The measurement of *NR3C1* methylation levels across various brain regions in these individuals will allow the consolidation of findings from different tissue types and could lead to effective and clinically

acceptable therapeutic interventions. Additionally, efforts must be made to isolate specific cell types, primarily those cells found in peripheral blood, in order to establish cell-specific methylation profiles (El-Sayed *et al.,* 2012).

Of particular note in the studies published to date is that there has been considerable heterogeneity in exactly where, and to what extent, within the *NR3C1* gene and its promoter regions, methylation has been assessed (Labonte *et al.*, 2012; Daskalakis and Yehuda, 2014). Much of the work that has been done has involved a specific location of the *NR3C1* gene itself, namely exon 1f and its promoter region incorporating the binding site for NGFIA. In the first of such studies Oberlander *et al.* (2008) looked at thirteen CpGs in an area comprising exon 1f, its promoter and a further section downstream of this, using Weaver et al's 2004 study as their main reference. McGowan *et al.* (2009), meanwhile, looked at 39 CpGs across exon 1f and its promoter. Perroud *et al.* (2011) looked at eight CpG sites across the exon 1f promoter and a further downstream region. Finally, Melas *et al.* (2012) analysed what are thought to be all of the 47 CpG sites throughout the main body and promoter region of *NR3C1*'s exon 1f. Although some effort has been made to correlate individual CpG sites across different studies this has not always been possible, and despite occasional agreement in which sites are differentially methylated no conclusive patterns have yet emerged. Hence, there would be benefit in researchers adopting a unified and comprehensive approach to the nucleotide sequence being assessed as well as the individual CpG sites within it. Given the extensive adoption so far of the nucleotide sequence from the Oberlander *et al.* (2008) study it is recommended that this continues to be used as the minimum and necessary sequence coverage for future *NR3C1* methylation studies in depressed patients with a history of early maltreatment.

Investigators have examined different functional correlates for the changes observed in *NR3C1* methylation. For example, Oberlander *et al.* (2008) used salivary cortisol measurements in the morning, following a stressor and in the evening, whereas McGowan *et al.* (2009) assessed *NR3C1* mRNA levels as well as NGFIA binding and NGFIA-induced transcription. Once again this heterogeneity of approaches has potentially impaired efforts to establish causal relationships. Although investigators have amassed a considerable

amount of evidence for an association between differential methylation and HPA axis function in humans, a causal relationship still needs to be fully established.

Lastly, a recurrent issue in studies examining childhood adversity is that of confounding factors relating to recall bias and the participant's current mental state. Ideally, long term follow-up of children up to and beyond the period of maximum risk of the development of psychiatric illnesses, with objective and detailed documentation of the reported maltreatment, will minimise this complication and simultaneously allow more effective exploration of the consequences of particular maltreatment categories.

Summary

Studies have continued to emerge that implicate hypermethylation of *NR3C1*'s exon 1f promoter occurring following early trauma with this being associated with HPA axis dysfunction and depression. Results have, however, been inconsistent at times and not without occasional controversy (Dyer, 2014). How much of the observed variation of findings is due to inter-subject variability in underlying pathophysiology, as opposed to experimental design, remains to be seen.

There is a need for researchers to adopt more consistent approaches to document the natural history of methylation patterns at individual CpG sites within *NR3C1* after early life adversity. This natural history will need to include other environmental factors such as age and diet (Mathers *et al.,* 2010; Murgatroyd and Spengler, 2011; Suderman *et al.*, 2012; Tyrka *et al.*, 2012; Bakulski and Fallin, 2014). A diet low in folate and high in methionine, for example, has already been associated with increased *NR3C1* methylation in mice (Sulistyoningrum *et al.*, 2012). The use of newer, more powerful technologies such as epigenome-wide association studies (EWAS) and single cell analysis are exciting but will bring challenges in terms of consistency, defining cell type, cost and data analysis (Plessy *et al.*, 2012; Callaway, 2014; Robinson *et al.*, 2014).

A significant challenge in the epigenetics of mental illness also continues to be the complex nature of these disorders and their aetiology (Caspi et al., 2003; Eaves et al., 2003; Bowes and Jaffee, 2013; Ehlert, 2013; Brown *et al.*,

2014; Castillo-Fernandez *et al.*, 2014; Kanherkar *et al.*, 2014). As is the case for most research in psychiatry there is the ever-present issue of difficulties with psychiatric nosology. Most authorities believe that the diagnostic category of "major depressive disorder" contains a heterogeneous collection of disorders with differing underlying pathophysiologies (Schmidt *et al.*, 2011). This will clearly hamper the interpretation of epigenetic data. This may be addressed by exploring the relationship between epigenetic status and endophenotypes such as those defined using the Research Domain Criteria initiative (Insel *et al.*, 2010; Casey *et al.*, 2013).

Studies on depression and *NR3C1* methylation are exciting in two main ways. Firstly they suggest a hitherto untapped approach that could help to synthesise a comprehensive and necessarily eclectic theory of depression, aiding our understanding of causal factors and identifying disease biomarkers. Secondly, they have the potential to point towards novel therapeutic targets since epigenetic changes are potentially reversible and therefore amenable to intervention, as has been seen in cancer, cardiovascular disease and neurological disorders (Heerboth et al., 2014; Sandhu *et al.*, 2015). Epigenetics offers a way to optimise the diagnosis, prognosis and treatment of depression. In doing so it could reduce depression's global burden whilst simultaneously providing a truly personalised medicine.

Conflict of Interests

The authors declare that there is no conflict of interests
regarding the publication of this paper.

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