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Ord, J., Fazeli, A. and Watt, P.J. (2017) Long-Term Effects of the Periconception Period on Embryo Epigenetic Profile and Phenotype: The Role of Stress and How This Effect Is Mediated. Advances in Experimental Medicine and Biology, 1014. pp. 117-135. ISSN 0065-2598

https://doi.org/10.1007/978-3-319-62414-3_7

Long term effects of the periconception period on embryo epigenetic profile and phenotype; (III) the role of stress and how this effect is mediated

James Ord*†, Alireza Fazeli†‡, Penelope J. Watt*

* Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN

† Department of Oncology and Metabolism, University of Sheffield, Level 4, Jessop Wing, Tree Root Walk, Sheffield S10 2SF

‡ Corresponding author (a.fazeli@sheffield.ac.uk)

Abstract

Stress represents an unavoidable aspect of human life, and pathologies associated with dysregulation of stress mechanisms – particularly psychiatric disorders – represent a significant global health problem. While it has long been observed that levels of stress experienced in the periconception period may greatly affect the offspring's risk of psychiatric disorders, the mechanisms underlying these associations are not yet comprehensively understood. In order to address this question, this chapter will take a 'top-down' approach, by first defining stress and associated concepts, before exploring the mechanistic basis of the stress response in the form of the hypothalamic-pituitary-adrenal (HPA) axis, and how dysregulation of the HPA axis can impede our mental and physical health, primarily via imbalances in glucocorticoids (GCs) and their corresponding receptors (GRs) in the brain. The current extent of knowledge pertaining to the impact of stress on developmental programming and epigenetic inheritance is then extensively discussed, including the role of chromatin remodelling associated with specific HPA axis-related genes, and the possible role of regulatory RNAs as messengers of environmental stress both in the intrauterine environment, and across the germ line. Furthering our understanding of the role of

stress on embryonic development is crucial if we are to increase our predictive power of disease risk and devise effective treatments and intervention strategies.

Key words: Stress, behaviour, periconception, hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoids, psychiatric disorders, microRNAs

1. Introduction

Psychiatric disorders such as anxiety, depression, schizophrenia, post-traumatic stress disorder (PTSD), and autism spectrum disorder (ASD) represent an enormous source of human suffering, and one of the leading causes of disability (Kalueff et al. 2014; Vos et al. 2015). While these conditions have a broad range of effects on our cognition, awareness, mood, and our perception of reality, they all implicate dysregulation of our biological stress response system: the hypothalamic-pituitary-adrenal (HPA) axis. Responding appropriately to stressful situations is integral to our survival, but the mechanism by which we do so can be impaired, to the detriment of both our psychological and physical health.

Both genetic and environmental factors are likely to contribute to the risk of developing psychiatric disorders. The environmental contribution to risk is not only affected by our own experiences, but, as a growing body of evidence now suggests, by our parents' experiences either during pregnancy or even before conception. Epidemiological data reveal that children whose mothers experience stress during pregnancy are at a higher risk of psychiatric disorders (Khashan et al. 2008), whilst rodent models show that gestational stress increases anxiety-like behaviour in adulthood (Lupien et al. 2009). The topic is further complicated by the interplay between stress and other aspects of our health; physical and psychological health often seem to go hand-in-hand, with patients suffering from psychological disorders at higher risk of detriment to their physical health, and vice-versa (Bradley and Dinan 2010). For example, while children who were in gestation during the Dutch famine of 1944-45 were found to be at an increased risk of metabolic syndrome (obesity and diabetes), their risk of psychiatric disorders, such as schizophrenia, also increased (Brown et al. 2000).

Due to the complexity of the human brain, in which psychiatric disorders manifest, to say that their underlying mechanisms and aetiologies are difficult to elucidate is a gargantuan understatement. Nevertheless, significant advances have been made in the past few decades in piecing together the developmental basis of the HPA axis and psychiatric disorders, using both epidemiology and model organism approaches. The following sections will address what we currently know about stress, its underlying mechanisms, how stress may be dysregulated in disease, and the crucial relevance of stress and the HPA axis to the periconception period and disease risk.

2. What is stress?

Although its definition is somewhat ambiguous in the biological literature, the consensus becoming increasingly accepted is that stress entails a state of disrupted homeostasis which is counterbalanced by adaptive mechanisms known as stress responses (Barton 2002; Chrousos 2009). As aspects of the biotic and abiotic environment are in constant flux, organisms must continually respond to environmental changes through homeostasis mechanisms. However, excessive stimuli pose a threat to homeostasis and require a stress response to restore balance – a process also referred to as allostasis (Schulte 2014). Stimuli which induce a stress response may be referred to as stressors.

The stress response underlies the body's often extraordinary ability to respond to unexpected danger, colloquially known as the fight or flight response (Sorrells et al. 2009). However, stress, by definition, is something we are not adapted to cope with excessively or repeatedly. Thus, excessive environmental stress impairs an organism's fitness due to 'wear and tear' referred to as allostatic load (Schulte 2014). The stress response is best geared towards restoring homeostasis in the face of single or acute stressors, while allostatic load accumulates in the face of prolonged or chronic stress, which may constitute several exposures, perhaps over a substantial duration of an organism's life cycle (Sorrells et al. 2009). Arguably the most significant source of chronic stress relevant to modern life is psychological, resulting from negative socioeconomic factors such as job insecurity, financial problems, bereavement and other personal struggles (Nargund 2015). The harsher end of chronic stress may include a prolonged state of real or perceived danger, such as domestic abuse, or severe resource detriment (i.e. famine).

This chapter will adhere to the concept of stress relating to activation of the HPA axis. It is to be considered distinct from oxidative stress (excessive exposure to reactive oxygen species), which is a ubiquitous mechanism underlying several biological processes, but does not necessarily implicate the HPA axis.

2.1 The Hypothalamic-Pituitary-Adrenal (HPA) axis

When the sensory systems detect a threat to homeostasis, a stress response is initiated in order to evoke endocrine and behavioural responses to enhance survival in the face of stress and ultimately restore homeostasis. Biologically, the stress response entails the initiation and regulation of a suite of endocrine pathways embodied by the hypothalamic-pituitary-adrenal (HPA) axis (Smith and Vale 2006; Bradley and Dinan 2010) (Fig. 1). As the name would suggest, the principal structures of the HPA axis are the hypothalamus (within the brain), pituitary gland (at the base of the brain) and adrenal glands (above the kidneys). In short, registration of a stress stimulus triggers a cascade of neuronal and endocrine events, culminating in the release of glucocorticoids (GCs) as the primary stress response, which interact with glucocorticoid receptors (GRs) to enact a variety of secondary adaptive responses.

[FIG. 1. HPA AXIS SCHEMATIC]

The name ‘glucocorticoid’ derives from early observations that the hormones are involved in glucose metabolism. The primary GC hormone in humans is cortisol, which initiates and regulates a suite of adaptive responses: it interacts with the central nervous system to induce changes in cognition and awareness, stimulates increased glucose production, providing readily available energy for responding to an immediate threat (i.e. fight or flight), and inhibits costly immune functions. Since the discovery of their immunosuppressive properties in the 1940s, GCs have provided useful anti-inflammatory drugs, which have been used to treat inflammatory diseases such as rheumatoid arthritis and asthma (Lupien et al. 2007). To regulate the stress response, cortisol also has an inhibitory effect on HPA activity in the hypothalamus, which establishes a negative feedback loop essential to healthy HPA axis functioning. The effects of cortisol and other GCs are mediated by the glucocorticoid receptor (GR), a cytosolic

protein complex composed of heat shock proteins (HSPs), and expressed in almost every cell type in the body. Following stress, GCs extensively occupy GRs, which enact transcriptional modifications either via binding with transcription factors, or as transcription factors themselves via direct interaction with glucocorticoid response elements (GREs). Thus, cortisol induces up or down-regulation of several genes, leading to the synthesis of enzymes responsible for glucose production, neurotrophic factors, and immunosuppressive factors. Cortisol dampens the stress response via the suppression of corticotrophin-releasing factor (CRF) and adreno-corticotropic hormone (ACTH) following GR binding in the hypothalamus and pituitary gland, respectively (Fig. 1).

Abnormal HPA axis functioning is associated with numerous pathologies, including both physical and psychiatric disorders. Both genetic and environmental factors may contribute to HPA axis dysfunction, which usually implicates imbalances of GCs, GRs, or both. Within the brain, GRs occur at high concentrations in the hippocampus, which is concerned with learning, memory, and attention (Lupien et al. 2007), and in the limbic system, which is responsible for emotion (Harris et al. 2013). Therefore, imbalances in levels of GCs or GRs have the potential to adversely affect attention span, emotional state, and other aspects of cognition. The association between GCs and psychiatric disorders first became evident in the 1950s through the increased incidence of psychosis in patients receiving GC therapy. These patients displayed gradually rising euphoria or dysphoria culminating in manic episodes, a condition which became known as “steroid psychosis” (Lupien et al. 2007). Since then, imbalances in GCs and GRs have been implicated in major depressive disorder (MDD) (Alt et al. 2010), schizophrenia (Bradley and Dinan 2010), posttraumatic stress disorder (PTSD) (Palma-Gudiel et al. 2015) and almost all anxiety disorders (Faravelli et al. 2012). The development of psychiatric disorders is frequently associated with chronic stress, such as childhood trauma, suggesting that HPA axis dysregulation may be induced by prolonged allostatic load at critical developmental stages (Heim and Nemeroff 2001; Lupien et al. 2009). This is possible due to the neuroplasticity of the early brain – its ability to reorganise its structure in response to intrinsic and extrinsic stimuli (Fenoglio et al. 2006; Cramer et al. 2011), and is now thought to be mediated by chromatin remodelling associated with GR. In rodents, for instance, repeated psychological stress leads to increased phospho-acetylation of histone

H3 in the hippocampus, but this is prevented by treatment with GR antagonists (Kolber et al. 2009). Furthermore, hypo-methylation of the NR3C1 gene (encoding GR) is found in PTSD patients (Palma-Gudiel et al. 2015), while long term alterations in DNA methylation in NR3C1 promoter regions has been suggested to mechanistically link MDD with childhood trauma (Alt et al. 2010).

Psychological illness is frequently associated with physical ill health. This may owe partly to the fact that the HPA axis does not only regulate the response to stress, but also influences many other bodily processes including cardiovascular function, energy provision, fat deposition, and immune responses (Kolber et al. 2009; Sorrells et al. 2009; Bradley and Dinan 2010). Thus, as well as affecting psychological health, disruption of HPA axis function through stress may have consequences for physical health. For example, excessive production of glucose resulting from overexposure to GCs may result in metabolic disorders such as type-2 diabetes (Bradley and Dinan 2010). Furthermore, the immunosuppressive properties of GCs leave the body open to infection in states of chronic stress. In mice, for instance, chronic psychological stress and subsequent increase in endogenous GCs induces downregulation of antimicrobial peptides, increasing the severity of a bacterial skin infection (Aberg et al. 2007). In contrast, however, in some cases of acute stress, GCs may also enhance the immune response in the central nervous system (Sorrells et al. 2009).

The HPA axis comprises an ancient mechanism which is largely conserved across the vertebrate subphylum, however, some inter-species differences (and similarities) are worthy of note. Importantly, rodents utilise corticosterone instead of cortisol as their primary GC hormone. Teleost fish possess an equivalent to the HPA axis called the hypothalamic-interrenal (HPI) axis, although the core stress response mechanism is virtually identical to its mammalian counterpart, and it is also noteworthy that fish, like humans, utilise cortisol as their principal GC hormone. In fact, some of the core endocrine components of the HPA axis are so deeply rooted in the evolutionary substrata that they form part of equivalent stress response systems in invertebrates (Ottaviani et al. 1994; Couto-Moraes et al. 2009).

[FIG. 2. EXAMPLES OF QUANTITATIVE BEHAVIOURAL AND PHISIOLOGICAL STRESS PHENOTYPES IN RODENTS AND FISH]

Because of the conserved nature of the stress response apparatus, methods for robustly quantifying behavioural and physiological stress responses have been developed in order to study HPA axis dysregulation in model organisms, including rodents and, more recently, fish. Rodent models of chronic stress typically entail a cocktail of stressful procedures administered daily, such as physical restraint or crowding, electric shock, exposure to fox odour, constant light, or loud noises (Takahashi et al. 1998; Aberg et al. 2007; Jensen Peña et al. 2012; Howerton et al. 2013). Cortisol or corticosterone concentrations in plasma or whole body samples (in the case of fish) can be quantified using enzyme-linked immunosorbent assay (ELISA) (Cachat et al. 2010), while several behavioural paradigms have been developed to quantify anxiety-like behaviour (Fig. 2). These include the open field test, which relies on rodents' innate aversion to an unfamiliar environment, and generally uses the time spent at the edge of the test arena (a behaviour called thigmotaxis) as a measure of anxiety (Prut and Belzung 2003), and the light-dark preference test, which relies on rodents' aversion to bright light (referred to as scototaxis), and uses time spent in darkness as a measure of anxiety (Bourin and Hascoët 2003; Arrant et al. 2013). Tests designed for rodents have been successfully adapted for use with zebrafish (Danio rerio) and other teleosts (Champagne et al. 2010; Ariyomo et al. 2013), while unique assays to measure anxiety-like behaviour in fish have also been developed, such as the novel tank diving test, which uses the depth of a fish in an unfamiliar tank as a measure of anxiety (Egan et al. 2009). For more extensive coverage of behavioural tests used to assess stress phenotypes in model organisms, readers are directed to Kumar et al. (2013) for rodents, and Stewart et al. (2012) for fish.

3. Stress dysregulation and periconception

We have so far defined stress, are aware of the core components of the stress response (the HPA axis) and the pathologies that may arise from its dysregulation, and how these can be translated into measurable phenotypes using model organisms. The remainder of this chapter outlines how HPA axis dysregulation, either by stress or other influences, during the periconception period may exert long term effects on disease risk, via epigenetic alterations enacted during embryonic (or gametic) development. Pathologies associated with maternal stress, malnutrition, and alcohol exposure during pregnancy

177 implicate HPA axis dysregulation, which may be induced via long term alterations to chromatin
178 structure, in turn mediated by complex placental transduction pathways. There is also evidence to
179 suggest that paternal stress influences embryonic development via epigenetic factors transmitted in
180 sperm, although much remains unknown regarding the underlying mechanisms. Fig. 3 presents a visual
181 summary of molecular and phenotypic effects of periconception stress which have been identified in
182 rodent models.

183 [FIG. 3. VISUAL SUMMARY OF PERICONCPETION STRESS]

184 3.1 Maternal influences

185 The prenatal period is now understood to be one of the most crucial stages of the human lifecycle in
186 terms of our future health and wellbeing, both physically and psychologically. Prenatal stress, which
187 may include domestic abuse, is associated with increased risk of adverse birth outcomes, such as
188 preterm birth (Lilliecreutz et al., 2016), and growth retardation (Cottrell and Seckl 2009), while
189 evidence has grown over the past few decades to link psychological stress during gestation with longer
190 term developmental outcomes. Depression during pregnancy, which affects up to 10% of women in the
191 UK (Vigod and Wilson 2016), with similar statistics reported in the US (Kinsella and Monk 2009;
192 Melville et al. 2010; Stewart 2011), has been shown to be a predictor of neurodevelopmental disorders
193 in children and adolescents, while maternal stress during the first trimester of pregnancy is associated
194 with increased risk of schizophrenia (Khashan et al. 2008), suggesting neurodevelopment is sensitive
195 to stress during this early window. Prenatal famine exposure, studied in the Dutch famine cohort, has
196 been associated with an increased risk of psychiatric disorders, including a two-fold increase in
197 schizophrenia and related conditions (Brown et al. 2000), while foetal alcohol exposure is associated
198 with later onset of depression and anxiety (Hellemans et al. 2010). Although several factors (e.g.
199 postnatal influences) may play a role in these observed effects, there is extensive interest in, and
200 growing evidence for, the impact of stress on prenatal development (particularly in relation to the HPA
201 axis) via alterations to in utero physiology and epigenetic programming (Kinsella & Monk 2009; Palma-

Gudiel et al. 2015). Such alterations undoubtedly involve complex interactions between the maternal environment, the placenta, and the developing embryo (Howerton et al. 2013).

GCs play several essential roles in embryonic development, particularly of the neural tissues (Harris and Seckl 2011), but overexposure to GCs resulting from stress has adverse consequences for prenatal development (Lupien et al. 2009). In rats, chronic stress during pregnancy increases corticosterone in both mother and foetus (Takahashi et al. 1998), which mediates increased anxiety-like phenotypes in adult offspring (Barbazanges et al. 1996; Lupien et al. 2009). GCs, which are employed for glucose production, are also increased in both mother and foetus during the state of chronic stress induced by under-nutrition (Blondeau et al. 2001), and as a result of alcohol exposure (Liang et al. 2011). Thus, HPA axis dysregulation resulting from overexposure to GCs may underlie pathologies associated with maternal stress and undernutrition (Brown et al. 2000), as well as foetal alcohol syndrome (Hellemans et al. 2010).

The molecular aetiology of developmental programming of the HPA axis in response to prenatal stress is likely to include epigenetic alterations to target chromatin, as chromatin organisation affects the levels of expression of associated gene sequences (Cottrell and Seckl 2009). Differential expression of three key placental genes have been implicated in prenatal stress: 11β-hydroxysteroid dehydrogenase type 2 (*11β*-HSD2), glucocorticoid receptor (NR3C1) (Conradt et al. 2013), and O-linked N-acetylglucosamine transferase (OGT) (Howerton et al. 2013). In addition, a host of regulatory RNAs have been implicated in developmental programming. However, these are likely only a few of the factors contributing to the byzantine dialect between the environment, placenta, and developing embryo, in which much remains to be elucidated.

11β-HSD2 regulates foetal GC levels by converting cortisol into inert cortisone, thus protecting the foetus from GC overexposure. Maternal stress, anxiety, and under-nutrition induce down-regulation of *11β*-HSD2, which has been shown to correlate with reduced birth weight, as well as HPA axis dysregulation and anxiety-like behaviour (Cottrell and Seckl 2009; Conradt et al. 2013). Similar outcomes are observed in homozygous knockout mice (*11β*-HSD2-/-) (Cottrell and Seckl 2009). In rats

exposed to chronic prenatal stress, foetuses possess reduced expression of *11β*-HSD2, and increased CpG methylation in the *11β*-HSD promoter region in hypothalamic tissue (Jensen Peña et al. 2012), while human mothers who report anxiety during pregnancy possess greater placental methylation of *11β*-HSD2 (Conradt et al. 2013). Collectively, the evidence suggests that *11β*-HSD2 is an important component of the molecular interface between the maternal environment and the developing foetus, and thus significant to the aetiology of stress-induced developmental pathologies.

NR3C1 is the gene encoding the glucocorticoid receptor (Conradt et al. 2013). Like GC, GRs are essential for normal development. For example, homozygous GR knockout mice die in the first few hours of life due to severely impaired lung development (Kolber et al. 2009). Likewise, reduction in NR3C1 expression by 30-50% in transgenic mice leads to exaggerated HPA axis responses to stress (Michailidou et al. 2008). There is now evidence to link this differential expression to targeted epigenetic reprogramming in response to prenatal stress. For example, mothers who report depression during pregnancy have higher methylation of placental NR3C1 (Conradt et al. 2013), while domestic abuse during pregnancy is significantly associated with methylation in the NR3C1 promoter in adolescent offspring (Radtke et al. 2011). A recent meta-analysis of human DNA methylation data from 977 individuals revealed that methylation of a single CpG site in the promoter region of NR3C1 was significantly associated with prenatal stress (Palma-Gudiel et al. 2015).

Another factor recently implicated in the link between prenatal stress and disease risk is O-linked N-acetylglucosamine (O-GlcNAc) transferase (Ogt). The enzyme is a key cellular regulator which modifies, by addition of O-GlcNAc, protein targets responsible for chromatin remodelling (e.g. RNA polymerases, histone deacetylases) (Howerton et al. 2013). Ogt also preferentially associates with TET proteins (regulators of DNA methylation state) in close proximity to CpG-rich transcription start sites (Vella et al. 2013). Maternal stress leads to reduced expression of placental OGT, and OGT-knockout mice develop HPA axis dysregulation characteristic of that induced by stress in early pregnancy (Howerton and Bale 2014). Deficiency of Ogt is hypothesised to underlie observations of male-biased risk of neurodevelopmental disorders, as it escapes X chromosome inactivation in the placenta and is

thus expressed at higher levels in females (Howerton et al. 2013). Furthermore, because O-GlcNAc is produced from glucose, Ogt is a potent sensor of cellular nutritional status, and is thought to be similarly responsive to other aspects of the environment (Zachara and Hart 2004; Love and Hanover 2005; Vella et al. 2013). Because of this, and because of its interaction with TET proteins and other factors associated with chromatin remodelling (Vella et al. 2013; Howerton et al. 2013), it is plausible that Ogt is a key mediator of stress-induced epigenetic alterations associated with *11β*-HSD and NR3C1.

In addition to, and very likely in conjunction with, DNA methylation, small noncoding RNAs are now believed to be essential regulators at the crossroads of genes, development, and environment. MicroRNAs (miRNAs) are small noncoding RNA molecules (~22 nucleotides) which modulate gene expression by either repressing translation or inducing degradation of target mRNAs (Hollins and Cairns 2016). They are abundant in the brain and exhibit brain region-specific expression patterns in response to acute and chronic stress in animal models (Hollins and Cairns 2016), suggesting they are important in neuroplasticity. Subsequently, there is now evidence that miRNAs are key mediators of stress-induced neurodevelopmental pathologies. In response to gestational stress, one study revealed that the brains of new-born mice exhibit differential expression of over 336 miRNAs (Zucchi et al. 2013). Several of these miRNAs are involved in neurodevelopment and have been implicated in psychiatric disorders, including miR-219, which is up-regulated in patients with schizophrenia. This differential miRNA expression was subsequently demonstrated to persist into the F2 generation, suggesting miRNAs may play a role in transgenerational programming of the oocyte (Yao et al. 2014), and thus may mediate epigenetic inheritance of disease risk. Interestingly, among the down-regulated miRNAs were miR-200b, which is implicated in uterine contractibility, and thus may provide a putative mechanistic explanation for preterm birth associated with gestational stress (Yao et al. 2014).

When considering long term implications of prenatal stress on HPA axis development, the neuroplasticity of the early postnatal brain (Cramer et al. 2011) must also be considered, as some lines of evidence suggest that alterations to HPA axis development in the prenatal period can be attenuated by intervention in the neonatal period. For example, rats exposed to handling during the preweaning

period exhibit permanent reductions in corticosterone secretion and GR expression (Welberg and Seckl 2008), and consequently, neonatal handling has been found to eliminate some of the adverse effects of foetal alcohol exposure, such as increased weight gain (Weinberg et al. 1995), and HPA axis hyperactivity (Ogilvie and Rivier 1997). However, subsequent experiments have produced conflicting results in this regard (Gabriel et al. 2000).

3.2 Paternal influences

The vast majority of literature on parental environmental influences on HPA axis development has focused on maternally-mediated effects. Understandably, given that humans are confined to the maternal environment for the first nine months, it was long thought that the paternal environment was of little importance. However, it has since become apparent that the spermatozoon provides to the embryo more than simply a haploid genome, and subsequently the paternal environment (particularly paternal stress) is becoming increasingly implicated in offspring disease risk, including HPA axis dysregulation.

Chronic psychological stress has long been perceived to be a potential risk factor in male infertility, and, although epidemiological studies have produced conflicting conclusions regarding the association, evidence is building that chronic psychological stress can significantly impair aspects of male fertility (Nargund 2015). Several clinical studies have now demonstrated an inverse relationship between psychological stress and semen parameters. For example, a recent analysis revealed an association between perceived stress or recent stressful life events, and a reduction in sperm concentration, motility, and normal morphology (Janevic et al. 2014). Mediated by GCs, stimulation of the HPA axis is now believed to have a direct inhibitory effect on the hypothalamic-pituitary-gonadal (HPG) axis, which drives key reproductive functions in both sexes, including spermatogenesis (Nargund 2015). Specifically, GCs inhibit the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, the downstream consequences of which include a reduction in testosterone, which is an essential regulator of spermatogenesis at several stages (Smith and Walker 2014; Nargund 2015).

In contrast to maternal effects, in which germ line-mediated effects are difficult to discern from in-utero effects on development, paternal effects on phenotype are more likely to represent the germ line transmission of environmental information. Germ line epigenetic inheritance has long been a puzzle due to the problem of erasure: the DNA methylation status of the parental genomes are re-set during the first few cell divisions, and thus it is widely thought that most alterations to methylation acquired during the parents' lifetime are erased (Cantone and Fisher 2013). However, acquired methylation changes may escape erasure in some cases, and other types of transmissible epigenetic factors are not subject to erasure, including a host of regulatory RNAs. Specifically, miRNAs have been heavily implicated, while much is yet to be deciphered regarding the possible role of sperm histones and protamines in the conveyance of environmental information.

A handful of studies have identified heritable alterations in measurable phenotypic aspects of HPA axis activity induced by paternal stress at different developmental stages. Male mice subjected to a chronic stress paradigm (maternal separation) in early life develop depression-like symptoms, as well as phenotypes consistent with dampened HPA axis responsivity such as reduced anxiety-like behaviour and reduced corticosterone in response to stress. These phenotypes were found to be inherited by the offspring, even when fertilisation was carried out in-vitro. RNAs were found to be integral to this environmental inheritance, as the injection of sperm RNA from traumatised males into normal zygotes recapitulated the observed phenotypes in the resulting pups (Gapp et al. 2014).

In addition to stress in early life, chronic stress experienced both in adolescence, and in during spermatogenesis (approx. 42 days in mice) in adulthood has been found to induce heritable alterations in measurable aspects of HPA axis activity. One dramatic example is the inheritance of a Pavlovian response, in which adult male mice were conditioned to associate the odour of acetophenone with an electric shock. The offspring of these mice, when presented with the same odour, exhibited a startle response without ever experiencing the electric shock. Olfr151, the gene encoding the odorant receptor for acetophenone was found to possess CpG hypomethylation in the sperm of both F0 conditioned and

F1 naïve males (Dias and Ressler 2014). However, whether the methylation state escaped erasure or was inherited by another mechanism is not clear.

A similar study (Rodgers et al. 2013) reported that male mice subjected to a 42 day chronic stress paradigm in either adolescence or adulthood sired offspring with dampened HPA axis activity, characterised by significantly lower corticosterone in response to stress compared to controls. These offspring also exhibited altered transcriptional profiles in the hypothalamus, including enriched expression of GC-responsive genes, and gene sets associated with chromatin remodelling (e.g. histone acetyltransferases). The researchers identified nine miRNAs exclusively expressed in the sperm of stressed males, the predicted targets of which included DNA methyltransferase 3a (DNMT3a), a critical regulator of de novo DNA methylation (Rodgers et al. 2013). Remarkably, in a subsequent study, these authors reported that inserting only these nine miRNAs into normal zygotes was sufficient to induce the same phenotype indicative of paternal stress (Rodgers et al. 2015). Similarly, it has been reported that injection of candidate miRNAs into normal zygotes recapitulates hereditary metabolic syndrome associated with paternal obesity (Grandjean et al. 2015). Thus, taken together, the evidence provides a strong case for miRNAs as a principal language of environmental inheritance.

Another interesting observation derived by comparing the published experiments is that similar hereditary HPA axis dysregulation occurs in response to paternal stress, irrespective whether stress is experienced in early life (Gapp et al. 2014), adolescence (Rodgers et al. 2013), or adulthood (Rodgers et al. 2013; Dias and Ressler 2014). This suggests that even though the phenotypes were induced in response to stress in different developmental stages, the underlying mechanism may be very similar if not the same. Extracellular vesicles are hypothesised to be important for intercellular communication via the exchange of genetic information in plants and animals (Mittelbrunn and Sánchez-Madrid 2012), and may be responsible for the transport of stress-induced miRNAs into sperm. It is also possible that stress-induced testosterone deficiency (Nargund 2015) may play a role in miRNA-mediated inheritance, as testosterone is known to regulate the expression of several miRNAs in Sertoli cells in the testes (Panneerdoss et al. 2012; Smith and Walker 2014). Interestingly, two of the nine stress-responsive

sperm miRNAs discovered by Rodgers et al (2013) (miR-25c and miR-375) are also regulated by testosterone, as shown using a mouse model of testosterone deprivation (Panneerdoss et al. 2012). MiR-375 well-characterised in terms of function, and is important for the development of the pancreas and pituitary gland, while little is known about the miR-25 family except that they are implicated in cardiac function (Wahlquist et al. 2014).

Another possibility is that stress may influence offspring phenotypes via post-translational modification to sperm chromatin structure, specifically histones and protamines. Chromatin undergoes extensive reorganisation during spermatogenesis, in which most histones are supplanted by protamines (Luense et al. 2016). Numerous unique protamine modifications, particularly acetylation and methylation, have been discovered in human and mouse sperm (Brunner et al. 2014), prompting the hypothesis that these decorations may play an important role in the epigenetic regulation of embryonic development following fertilisation, and furthermore may represent mediators of germline epigenetic inheritance (Luense et al. 2016). Although most paternal histones and protamines are believed to be replaced by maternally-inherited histones soon after fertilisation (Cantone and Fisher 2013), sperm histone marks retained at fertilisation have recently been reported to be essential for correct gene expression in Xenopus embryos (Teperek et al. 2016). There is still very little known regarding sperm histone and protamine post-translational modifications, including the extent to which they may be subject to external environmental influences, and thus more attention is needed in this area of research.

Although the underlying mechanisms remain elusive, it is clear that environmentally-induced reprogramming occurs not just in the developing embryo, but in developing germ cells. The observation that the same phenotypes induced by paternal stress in early life and adolescence is also induced by stress during spermatogenesis suggests that, rather than resulting from long-term alterations to germ cell precursors, modifications to maturing germ cells occur transiently in response to long term alterations to HPA axis functionality. If this is the case, effective therapy and restoration of normal HPA axis function may halt or at least reduce the modification of maturing germ cells, preventing the inheritance of pathologies. Alternatively, if miRNAs do indeed constitute the principal language of

environmental inheritance, blocking those miRNAs up-regulated by paternal stress (or supplementing those down-regulated) may prevent this differential expression from manifesting in pathologies in the offspring.

So far, paternal effects mediated by miRNAs have been identified only in rodent models, with some evidence of similar mechanisms existing in Caenorhabditis elegans (Grossniklaus et al. 2013). There is evidence that environmental exposures can influence the miRNA content of human sperm (Marczylo et al. 2012), and it has been suggested that paternal trauma or experience of violence, such as in the case of war veterans and holocaust survivors, may be paternally transmitted and influence offspring mental health (Vaage et al. 2011). However, little evidence has emerged from epidemiological studies to suggest that such paternal exposures are transmissible down the human germ line (Yehuda et al. 2001; Vaage et al. 2011), and such associations may be more likely to arise due to behavioural influences on children, rather than epigenetic transmission. Whether such mechanisms exist in distantly related vertebrates, such as fish, is not known, although non-genetic transgenerational phenomena associated with environmental stress have been observed in teleost fish (Miller et al. 2012), and miRNAs are known to play an essential role in teleost spermatogenesis (Babiak 2014). If the mechanisms of inheritance in other vertebrates are similar to those being delineated in rodents, it would hint at the evolutionary significance of miRNA-mediated environmental inheritance, and it is possible that the mechanism may hold an ancient adaptive function (Grossniklaus et al. 2013).

4. Conclusions and future directions

The aetiologies of psychiatric disorders remain frustratingly elusive, making efforts to devise effective treatments still difficult. However, recent studies in both humans and animal models have shown promise in uncovering the molecular basis of these conditions, including altered epigenetic states resulting from exposure in early life, gestation, or pre-conception. As high-throughput sequencing technologies and other molecular tools become more affordable and accessible, it will be possible to further address knowledge gaps pertaining to the mechanisms underlying long term effects of periconception stress. For instance, although specific chromatin marks and regulatory RNAs have been

implicated in long term effects of parental stress, there remain several such entities, such as long noncoding RNAs (lncRNAs), the functions of which we know very little about (Morris and Mattick 2014).

A well-established toolset already exists for studying stress dysregulation in model organisms, which continue to help further our understanding of this complex set of processes. There is now increasing interest in non-mammalian models, specifically zebrafish, which present an increasingly attractive avenue for the exploration of periconception stress. The rapid life cycle and easily manipulated transparent embryos of the zebrafish (D. rerio) have already made them one of the most powerful vertebrate tools available to embryologists, and there exists a well-developed toolset for studying their behavioural and physiological stress phenotypes (Cachat et al. 2010; Stewart et al. 2012). Zebrafish may also present a unique, high-throughput model for epigenetic effects associated with spermatogenesis, the duration of which is a mere six days in this species (Leal et al. 2009).

In addition, having uncovered previously unknown mechanisms of environmental inheritance in model organisms, further attention may be directed to epidemiology to determine the significance of these mechanisms in human populations. Unique miRNA profiles have already been identified in the sperm of smokers versus non-smokers (Marczylo et al. 2012), suggesting other environmental influences, particularly stress, may affect gametic chromatin, with consequences for subsequent embryos. There is therefore a need to characterise miRNAs from gametes derived from humans suffering from chronic stress, as these may provide valuable molecular markers for risk of HPA axis dysregulation in subsequent generations.

In conclusion, an improved mechanistic understanding of environmental pre-disposition to HPA axis-related pathologies will have major benefits to public health, in the interests of both treatment and prevention. Increased knowledge of molecular pathways underlying disease risk may provide important biomarkers, such that those already at risk of psychiatric disorders may be identified, enabling early intervention to minimise long term suffering. Increased knowledge of disease processes may also pave the way for the development of therapeutic agents to counteract the adverse effects of parental stress on

offspring disease risk. Finally, increased awareness of environmental influences on development will help to further inform human lifestyles and behaviour, such that risk to subsequent generations is minimised.

FIGURE LEGENDS

Fig. 1. Schematic diagram of the hypothalamic-pituitary-adrenal (HPA) axis. Upon registration of a stress stimulus by sensory neurones, information is relayed to the paraventricular nucleus (PVN) in the hypothalamus. The PVN continuously synthesises corticotropin-releasing factor (CRF) which, in response to sensory stimuli, is secreted into portal blood vessels which lead to the pituitary gland. Here, binding of CRF to its receptors induces the release of adreno-corticotropic hormone (ACTH), which enters the systemic circulation. Circulating ACTH reaches the adrenal cortex, situated along the perimeter of the adrenal gland, and upon reception stimulates the production and release of glucocorticoids (GCs) from the adrenal gland. GCs interact with glucocorticoid receptor (GR) to enact secondary adaptive responses, as well as the inhibition of the HPA axis via negative feedback. Plus signs: stimulatory effects, minus signs: inhibitory effects.

Fig. 2. Examples of quantitative behavioural and physiological stress phenotypes in rodents and fish. Strong anxiety-like behaviour (e.g., excessive thigmotaxis, scototaxis, or time spent in the lower region of a novel tank) is generally exhibited in response to HPA (mammals) or HPI (fish) axis activation by a stressor. Abnormal levels of anxiety-like behaviour detected using these measures may be indicative of dysregulation of the HPA or HPI axis, which may result from chronic stress. Following behavioural testing, cortisol may be extracted from serum or whole body samples and quantified using ELISA.

Fig. 3. Summary of molecular pathways altered by chronic stress in the periconception period, and phenotypic effects in offspring. Chronic maternal stress in the prenatal period induces down-regulation of *11β*-HSD2 (Conradt et al. 2013), NR3C1 (Cottrell and Seckl 2009; Conradt et al. 2013), and OGT (Howerton and Bale 2014) in the placenta and / or foetus, and postnatal phenotypes indicative of HPA axis hyperactivity (increased glucocorticoids and anxiety-like behaviour) (Lupien et al. 2009). Chronic

paternal stress in early life (Gapp et al. 2014), adolescence, or adulthood (Rodgers et al. 2013) alters sperm RNA composition, and induces phenotypes indicative of suppressed HPA axis activity (decreased glucocorticoids and anxiety-like behaviour) in subsequent offspring. Insertion of sperm RNAs from stressed males into normal zygotes by microinjection recapitulates the paternal stress phenotypes in resultant pups (Gapp et al. 2014; Rodgers et al. 2015).

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