Mechanisms of Non-Genetic Inheritance and Psychiatric Disorders

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Inheritance is typically associated with the Mendelian transmission of information from parents to offspring by alleles (DNA sequence). However, empirical data clearly suggest that traits can be acquired from ancestors by mechanisms that do not involve genetic alleles, referred to as non-genetic inheritance. Information that is non-genetically transmitted across generations includes parental experience and exposure to certain environments, but also parental mutations and polymorphisms, because they can change the parental 'intrinsic' environment. Non-genetic inheritance is not limited to the first generation of the progeny, but can involve the grandchildren and even further generations. Non-genetic inheritance has been observed for multiple traits including overall development, cardiovascular risk and metabolic symptoms, but this review will focus on the inheritance of behavioral abnormalities pertinent to psychiatric disorders. Multigenerational non-genetic inheritance is often interpreted as the transmission of epigenetic marks, such as DNA methylation and chromatin modifications, via the gametes (transgenerational epigenetic inheritance). However, information can be carried across generations by a large number of bioactive substances, including hormones, cytokines, and even microorganisms, without the involvement of the gametes. We reason that this broader definition of non-genetic inheritance is more appropriate, especially in the context of psychiatric disorders, because of the well-recognized role of parental and early life environmental factors in later life psychopathology. Here we discuss the various forms of non-genetic inheritance in humans and animals, as well as rodent models of psychiatric conditions to illustrate possible mechanisms.
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INTRODUCTION

The traditional view that Mendelian inheritance can fully explain heritability is no longer satisfactory (Bonduriansky, 2012). Several lines of evidence indicate the existence of multiple mechanisms that underlie the acquisition and transmission of traits across generations (Figure 1). Non-genetic inheritance could partly explain 'missing heritability', the discrepancy between the high heritability of complex disorders, and the small number of identified risk genes associated with those disorders (Manolio *et al*, 2009; Slatkin, 2009). In principle, non-genetic inheritance comprises all parent–offspring mechanisms of inheritance, except the transmission of alleles (DNA sequence variants). While non-genetic inheritance is typically associated with parental experience or environment, it is important to acknowledge that parental mutations and genetic variability, by altering the parental 'intrinsic' environment, can also result in a

phenotype that is transmitted non-genetically to the genetically unaffected offspring (Figure 1).

The most plausible mechanism of transmitting information across generations is via the gametes, referred to as 'gametic non-genetic inheritance' (Figure 1). Although this mode of non-genetic inheritance is typically believed to be transmitted by epigenetic signatures, such as secondary DNA and chromatin modifications, identifying epigenetic alterations in the germline, that are propagated to the embryo and adult offspring, remains elusive. Nevertheless, carriers of parental information in the gametes may include RNA and prions, in addition to DNA methylation and chromatin modifications. Recent data also demonstrate that non-gametic inheritance occurs via parental hormones, immune molecules, and even microorganisms (Howerton and Bale, 2012) (Figure 1). Still other non-gametic mechanisms of inheritance are social or behavioral transmission of phenotypes, a topic extensively covered in previous reviews (Meaney, 2001; Champagne and Meaney, 2007; Cameron *et al*, 2008), as well as in this issue.

In contrast to genetic inheritance, non-genetic inheritance typically affects only a limited number of generations, from a single to a few. Although an evolutionary role for non-genetic inheritance has been implicated (Danchin *et al*, 2011;
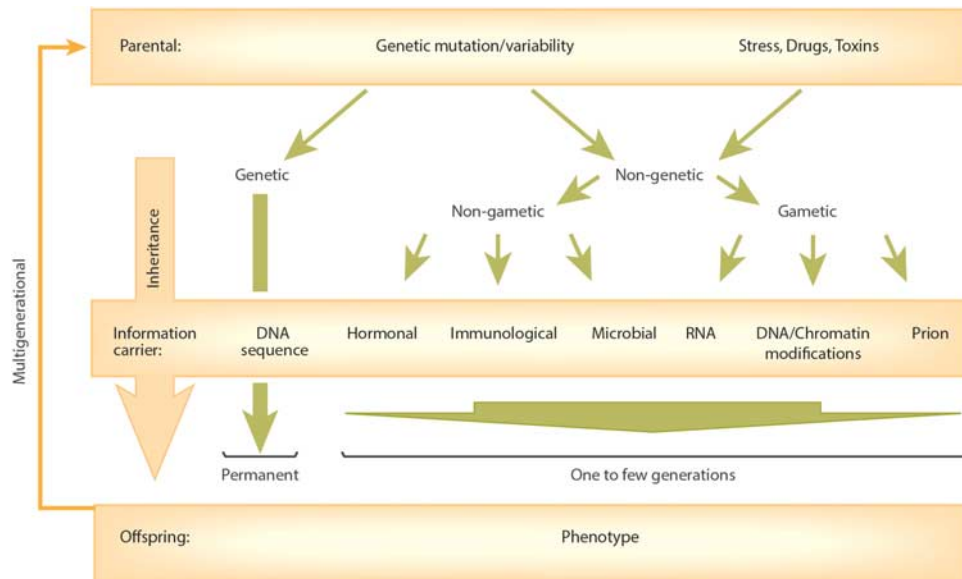
Figure 1. Multiple pathways of transmitting non-genetic information across generations.

Day and Bonduriansky, 2011; Hunter *et al*, 2012), its limited transmission is more consistent with an adaptive, also called programming, function (Agrawal *et al*, 1999; Dantzer *et al*, 2013). According to this hypothesis, non-genetic inheritance would exert its effects by providing information on the parental environment to the offspring (and to a few generations of descendants) during development to increase their fitness (eg, survival and reproduction). This idea can be extended to include disease that occurs either when adaptive programming is disrupted (for example by a maternal genetic mutation) or when the response initially facilitates survival, but carries consequent tradeoffs manifested as diseases (Frankenhuis and Del Giudice, 2012).

EPIDEMIOLOGICAL EVIDENCE FOR THE PROPAGATION OF NON-GENETIC BEHAVIORAL EFFECTS ACROSS GENERATIONS IN HUMAN

Effect of the Environment

It is now well established that maternal malnutrition, infection, stress, and psychiatric conditions are associated with an increased incidence of behavioral abnormalities in children manifested at adolescent/adult age. For example, children of women pregnant during the Dutch famine in WWII, besides being smaller than normal at birth, had behavioral abnormalities (Hoek *et al*, 1998; Painter *et al*, 2005). Similarly, adult children of Holocaust survivors have increased risk for anxiety and higher levels of psychological distress (Scharf, 2007; Gangi *et al*, 2009). Also, maternal infection during pregnancy, especially when it requires hospitalization, has repeatedly been associated with a higher incidence of autism and schizophrenia in the progeny (Patterson, 2009; Brown, 2012; Zerbo *et al*, 2013).

Parental effects are not limited to the first generation (F1) offspring and can be extended to the grandchildren (F2) (Lumey *et al*, 2011). Studies showed a higher than normal vulnerability of the adult grandchildren of Holocaust survivors to psychological distress (Sigal *et al*, 1988; Scharf, 2007). Similarly, a higher incidence of poor health in later life of grandchildren of mothers who suffered from the Dutch famine during pregnancy was also reported (Painter *et al*, 2008).

Effect of Parental Psychiatric Conditions

A number of reports have found a direct correlation between parental psychiatric disease and the presence of psychopathology in the offspring. Although genetic inheritance is difficult to exclude in these studies, heritability is higher than expected based on known gene variants in complex diseases (see missing heritability above), indicating that non-genetic inheritance may account partly for the missing components. For example, maternal posttraumatic stress disorder (PTSD) increases the risk for PTSD in the offspring (Yehuda *et al*, 2008; Roberts *et al*, 2012) and maternal antenatal anxiety is associated with behavioral problems in young children (O'Connor *et al*, 2002) as well as with hypothalamo–pituitary–adrenal (HPA) axis dysregulation and depressive symptoms in adolescent children (Van den Bergh *et al*, 2008). Similarly, maternal depression and social phobia are associated with increased incidence of child psychopathology (Tillfors *et al*, 2001; Weissman and Jensen, 2002; Halligan *et al*, 2004; Huizink *et al*, 2004; Halligan *et al*, 2007; Brummelte and Galea, 2010; Murray *et al*, 2010). Furthermore, another study investigated the effect of depression on prepubertal psychopathology in two generations of offspring, particularly on anxiety (anxiety, in contrast to depression, often has a prepubertal onset), and

found that grandparent and parent major depression is associated with prepubertal anxiety (Weissman and Jensen, 2002). The highest risk for anxiety in the grandchildren was associated with both parental and grandparental depression, while grandchildren with only parental, but not grandparental depression, had the lowest risk, suggesting that grandparental depression has a higher impact on children anxiety risk than depression in the parent.

Limitations in Studying Non-Genetic Inheritance in Human

Information about the behavioral effects of the environment on consecutive generations in humans is limited and so far spans only three generations. In addition, all human studies are correlative, precluding the study of the underlying mechanism of non-genetic inheritance. For example, it is difficult, if not impossible to determine whether the information transfer between parents, children, and grandchildren is based on behavioral interactions or the transfer of hormones or other substances via the placenta or milk, or whether it is mediated by the inheritance of epigenetic marks via the gametes. Distinguishing between these possibilities and identifying the contribution of non-genetic inheritance to phenotypes require animal models (discussed below).

An additional complication is that the child or grandchild condition can be due to the shared exposure with the parents and grandparents that would not qualify for non-genetic inheritance. This confound can be excluded if the parental and grandparental conditions are known to be terminated before the birth of the child.

NON-GENETIC INHERITANCE IN ANIMAL MODELS

Non-genetic inheritance across single or multiple generations was originally observed in animals, both in the wild (Agrawal et al, 1999) and in the laboratory (Harper, 2005; Whitelaw and Whitelaw, 2008; Bohacek et al, 2013). Rodents in particular, due to their widespread use and their well-established genetics and embryo transfer techniques, are suitable models to study non-genetic inheritance at the mechanistic level.

Effect of Stress, Abused Drugs and Toxins Across Generations

The initial trigger of non-genetic inheritance is often an external environmental factor, such as limited calorie intake, infection, stress, drugs of abuse and toxins, resulting in an adverse gestational environment that leads to behavioral abnormalities in the offspring (Veenema et al, 2008; Chen et al, 2011). Many of these effects are multigenerational and here we discuss examples that report on transmission to at least the F2 generation.

Parental and postnatal stress is a well known factor in causing behavioral abnormalities in the offspring. For

example, stress during pregnancy results in increased activity in the F2 generation (Wehmer et al, 1970). Maternal malnutrition and synthetic glucocorticoid administration alter HPA axis functions in both the F1 and F2 generations, that can lead to stress-related behavioral phenotypes (Kostaki et al, 2005; Bertram et al, 2008). In some models, stress exposure of the female results in transmission to the F2 generation, but only through F1 males (Morgan and Bale, 2011; Saavedra-Rodriguez and Feig, 2012), suggesting male gamete-mediated F1 to F2 transmission. Maternal separation during early postnatal development results in behavioral deficits in the F1 offspring that are transmitted to the F2 generation through females (Weiss et al, 2011). Finally, mice exposed to chronic social stress during adolescence and early adulthood exhibit persistent behavioral alterations, including enhanced anxiety and social deficits (Saavedra-Rodríguez and Feig, 2013). Although both exposed mothers and fathers can transmit the altered behaviors to their female F1 offspring, only F1 fathers transmit them to their F2 and F3 daughters.

Drug abuse is another environmental trigger of multigenerational behavioral effects. The first reports that drugs may have behavioral effects beyond the exposed individual and across generations came from studies showing that offspring of males exposed to opiates, cocaine, and alcohol have increased anxiety and impaired spatial learning (Abel et al, 1989; Ledig et al, 1998; He et al, 2006). More recently, it was demonstrated that offspring of female rats exposed to morphine during adolescence have alterations in recognition memory, anxiety-like behavior, and social interaction, as well as in opiate sensitivity and tolerance (Byrnes, 2005a, b, Byrnes, 2008; Byrnes et al, 2011). Interestingly, some of these effects were further transmitted to the F2 generation (Byrnes et al, 2013). A similar heritable phenotype, resulting from the self-administration of cocaine, was also described in rats (Vassoler et al, 2013). The phenotype included delayed acquisition and reduced maintenance of cocaine self-administration in male, but not female, offspring of sires that self-administered cocaine. Whether this phenotype is transmitted to further generations was not reported. Similarly, parental exposure to Δ^9-tetrahydrocannabinol (THC), the main psychoactive component of cannabis, resulted in increased work effort to self-administer heroin in the F1 generation (Szutorisz et al, 2014). As both parents were exposed to THC, whether the transmission occurs via male or female is not known.

Some environmental toxins, such as endocrine disruptors, are able to affect not only the exposed individual but the subsequent, and even consecutive, generations. Exposure during gestation to diethylstilbestrol (DES), an agent with estrogen agonist activity, produces reproductive tract dysfunctions in both the F1 and F2 generations (Newbold et al, 2006). The anti-androgenic endocrine disruptor flutamide causes testicular abnormalities in the F1 and skeletal developmental problems in the F2 generation (Anway et al, 2005). The effect of another endocrine disruptor, the fungicide vinclozolin, which has anti-androgenic endocrine actions, is

extended to the F3 generation and beyond (Anway et al, 2005; Anway et al, 2006). Exposure of rats to this toxin during the second week of pregnancy, which is the critical period of gonadal sex differentiation in the fetus, produces impaired spermatogenesis in the F1 and up to the F4 generation. Interestingly, additional phenotypes, including decreased anxiety in F3 males and increased anxiety in F3 females were also reported (Skinner et al, 2008). The different behavioral effects in males and females are not unusual and reflect the gender-specific manifestations of non-genetically acquired phenotypes, similarly to those that are caused by genetic or direct environmental factors (McCarthy et al, 2012).

Maternal Mutations and Genetic Variability as Intrinsic Environmental Factors in Non-Genetic Inheritance

While the previous section discussed the effect of the external environment on the parents, and in turn, on the offspring, this section summarizes published data on how parental genetic variability, as an intrinsic factor, can alter the behavior of their offspring via a non-genetic mechanism (ie, ancestral genotype effect). As single genetic mutations are easier to control and standardize within and across experiments, in addition to the fact that they represent a more specific starting point in mechanistic studies than the external environment, mutation-based models may provide an advantage in deciphering the non-genetic transfer of information from mutant parents to genetically unaffected offspring.

It was reported that the genetically wild-type (WT) offspring of Dnmt1a (DNA methyltransferase) and SMAR-CA5 (SWI/SNF related, matrix associated, actin-dependent regulator of chromatin) heterozygote fathers exhibit transcriptional changes and chromosome ploidy in adulthood (Chong et al, 2007). As these proteins are essential in DNA methylation and chromatin remodeling, their haploinsufficiency may disrupt epigenetic reprogramming in the male gametes resulting in the offspring phenotypes. Also, we reported that the genetic inactivation of one or both alleles of the serotonin $(5\text{-HT})_{1A}$ receptor $(5\text{-}HT_{1A}R)$ results in an anxiety-like phenotype in the mother, characterized by the avoidance of open areas in preference for safe areas, reduced exploration in large spaces, and increased reactivity in unavoidable stress situations. Furthermore, these three hallmarks of anxiety are transmitted to their WT F1 offspring. Some of the anxiety-related behaviors were also transmitted to the F2 and F3 generations (Laird and Toth, unpublished). Finally, a recent study showed that a hypomorphic mutation in the enzyme methionine synthase reductase (Mtrr) in the dams, that is necessary for the utilization of methyl groups from the folate cycle, results in various congenital malformations, including neuronal tube defects in the maternal grand progeny (Padmanabhan et al, 2013). Some of these defects persisted for five generations.

Genetic/epigenetic variability in the parents can also be a source of non-genetic transmission. Individual females with high and low maternal care (defined as individuals' 1 SD above and below the group mean, respectively) were selected from an outbred rat population and the anxiety phenotype of their offspring was followed. While the offspring of mothers with low level of care attain anxiety, when they are cross-fostered at birth to mothers with high level of care acquire a low level of anxiety (Caldji et al, 1998; Liu et al, 2000). Although it is not entirely clear whether the variability in maternal care was due to genetic, environmental, or stochastic epigenetic differences, these experiments indicate that the transmission is non-genetic. This cycle of non-genetic inheritance is repeated across generations (Francis et al, 1999). Another similar experiment utilized two inbred strains of mice with differing anxiety levels. Embryo transfer and cross-fostering (Figure 2) demonstrated the non-genetic transmission of high anxiety phenotype, characteristic for the BALB strain, to B6 offspring, that normally exhibit low anxiety, during the period that encompassed both gestation and early postnatal life (Francis et al, 2003).

MECHANISMS UNDERLYING THE PROPAGATION OF NON-GENETIC EFFECTS ACROSS GENERATIONS

The transmission of parental experience across generations could be based on several different mechanisms. The most plausible mode of information transfer is via the gametes. However, parental experience can be passed through the placenta and milk to the fetus/neonate via biologically active molecules whose effects reconstitute the parental phenotype. This process is repeated in the next generation, perpetuating the parental phenotype via a non-gametic mechanism. Embryo transfer and/or cross-fostering experiments can distinguish non-gametic prenatal and postnatal transfer of information from that of gamete-mediated non-genetic inheritance (Figure 2). Offspring, derived from matings of unaffected parents, should exhibit the phenotype when transferred as embryos or cross-fostered as newborns to affected mothers/parents, if the transmission occurred during gestation or postnatally, respectively (Figure 2a). For example, WT mice implanted as 1-day-old embryos into 5-$HT_{1A}R$-deficient mothers and then cross-fostered at birth to WT mothers exhibited anxiety mentioned above, indicating that the non-genetic transfer occurred during gestation (Gleason et al, 2010). Similarly, embryo transfer experiments demonstrated that the effects of the maternal Mtt mutation are transferred to their WT offspring nongenetically during the gestation period (Padmanabhan et al, 2013). These experiments also excluded the possibility that the offspring phenotype was caused by 'spurious' gene mutations or genetic polymorphisms; unequivocally demonstrating non-genetic inheritance. In contrast, offspring generated by in vitro fertilization (IVF) or from early
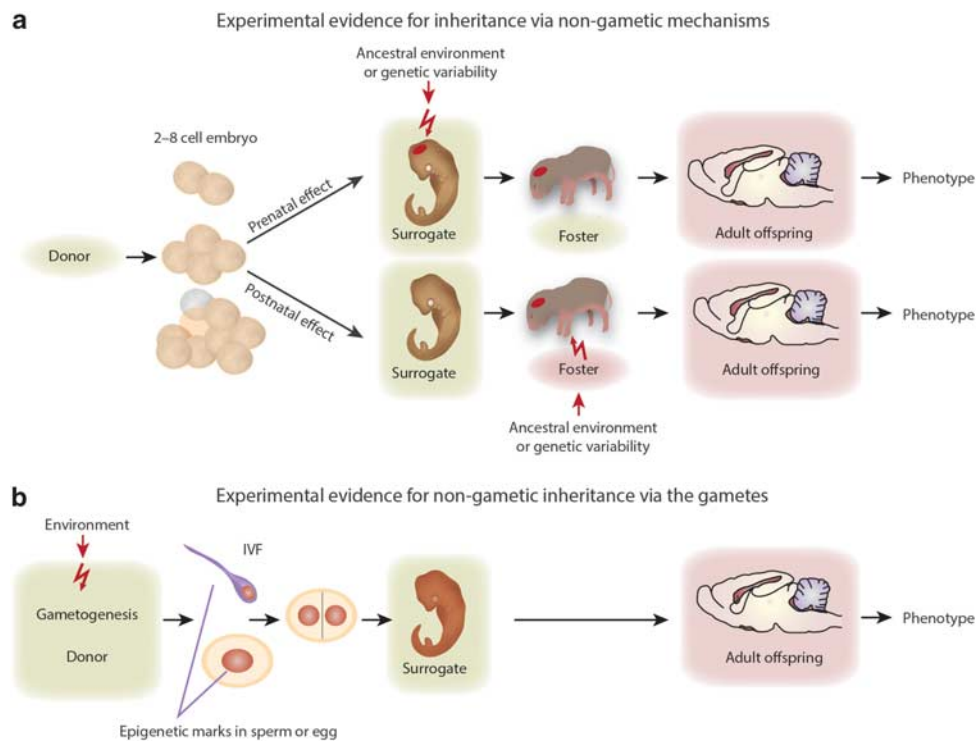
Figure 2. Embryo transfer, *in vitro* fertilization (IVF) and cross-fostering can distinguish between gametic and non-gametic prenatal and non-gametic postnatal mechanisms of non-genetic inheritance.

embryos derived from affected parent(s) and implanted to unaffected mothers should display the phenotype if the transmission is gametic (Figure 2b).

Environmental Effects Perpetuated via 'Non-Gametic' Transmission during the Prenatal and Early Postnatal Period

Molecular substrates carrying information from mother to offspring. Somatic cells, for example neurons in the developing brain, can acquire properties during fetal or early postnatal life from their parents, in particular from their mother (but also from their father), via the transfer of bioactive molecules. These bioactive substrates include hormones, cytokines, immune cells, and even parental microorganisms (Howerton and Bale, 2012) (Figure 1). For example, stress during pregnancy results in increased levels of glucocorticoids that can alter the HPA axis and the stress response of the offspring (Koehl *et al*, 1999). Although the placenta expresses the enzyme 11-hydroxysteroid dehydrogenase (HSD), which under normal conditions converts the glucocorticoids into inactive products (Reynolds, 2013), high levels of maternal circulating glucocorticoids can reach the fetal brain (Zarrow *et al*, 1970). Moreover, stress can reduce placental HSD, which further facilitates maternal glucocorticoids to reach the fetus (Mairesse *et al*, 2007). Increased maternal glucocorticoid levels sensitize the various neuroendocrine systems, most significantly the HPA axis, increasing stress-induced glucocorticoid secretion (Peters, 1982; Henry *et al*, 1994) and prolonging their

secretion (Fride *et al*, 1986; Maccari *et al*, 1995; Barbazanges *et al*, 1996). In general, the neuroendocrine changes are accompanied by increased anxiety and depression-like behaviors (Vallée *et al*, 1997), altered locomotor activity (Maccari *et al*, 2003), and impaired spatial learning (Ishiwata *et al*, 2005) in the offspring. As the physiological adaptation of the offspring to prenatal stress recapitulates the maternal stress response, the effect may be further transmitted (Matthews and Phillips, 2010). Indeed, glucocorticoid administration during gestation resulted in altered HPA function in F2 females and hippocampal glucocorticoid feedback in both F2 males and females (Iqbal *et al*, 2012).

Cytokines could also transmit information from mother to offspring, and perhaps to additional generations. For example, excess levels of maternal interleukin 6 can reprogram the offspring brain and lead to behavioral abnormalities that resemble some of the symptoms of autism spectrum disorders (Smith *et al*, 2007; Patterson, 2009; Malkova *et al*, 2012). Also, we have shown that maternal tumor necrosis factor α (TNFα) levels alter the spatial memory of the offspring (Liu *et al*, 2014).

Still another potential pathway of the mother to offspring communication involves microorganisms. At birth, the gastrointestinal tract (GI) of neonates is sterile and is gradually colonized through exposure to the maternal microbiota (vaginal, fecal, oral, and dermal) (Curtis and Sloan, 2004). Colonization occurs in a succession by various bacterial species that leads to a final mature microbiota whose composition has a significant effect on brain functioning and behavior. For example, maternal/perinatal

stress reduces Lactobacillus and Bifidobacterium species in the gut microbiome and results in increased stress reactivity and anxiety in the offspring, while ingestion of Lactobacillus has the opposite effect (Sudo *et al*, 2004; Gareau *et al*, 2007; Desbonnet *et al*, 2010; Bravo *et al*, 2011). The gut microbiome may communicate with the brain via the immune system, the vagus nerve, or by changing the permeability of the GI track affecting the adsorption of food and bacteria-derived products (Fåk *et al*, 2008; Bravo *et al*, 2011; Al-Asmakh *et al*, 2012; Hooper *et al*, 2012; Nicholson *et al*, 2012). As stress and environmental influences alter the composition of the gut microbiome and because the offspring gut microbiome is derived from their mothers, microbiome-associated psychopathology could be perpetuated across generations.

Relevance to psychiatric disorders. Similarly to the results obtained in animal experiments, exposure to excess glucocorticoids during prenatal life in humans alters the functioning of the HPA axis and the stress response of the offspring. For example, paired maternal–fetal and maternal–cord blood samples show positive correlation indicating that high cortisol in the mother is associated with high cortisol in the fetus and newborn (Gitau *et al*, 2004; Smith *et al*, 2011). High mid/late gestation maternal cortisol levels are also correlated with higher cortisol response to stress in the newborn. The increased HPA activity, as a result of higher than normal intrauterine cortisol, persists into adulthood (Phillips *et al*, 2000). Intrauterine exposure to excess maternal cortisol can be due not only to maternal stress but also to loss of function mutations in HSD (Dave-Sharma *et al*, 1998). Still another example of environmentally induced exposure to excess cortisol in human occurs when pregnant woman at risk of preterm delivery are treated with synthetic glucocorticoids to promote fetal lung development. Although studying the behavioral effects of excess prenatal glucocorticoid is complicated by confounds such as premature birth, accumulating data suggest permanent behavioral problems in these children that include increased distractibility and inattention (French *et al*, 1999), impaired cognitive development and narrative memory, and increased risk for attention deficit hyperactivity disorder (Räikkönen *et al*, 2009; Bergman *et al*, 2010). This process may be perpetuated across generations and could explain the higher vulnerability of adult grandchildren of mothers exposed to extreme stress to psychological distress (Sigal *et al*, 1988; Scharf, 2007) and depression (Weissman and Jensen, 2002).

Non-Genetic Inheritance via the Gametes

It is typical to think of non-genetic inheritance as gametic transmission of traits with an epigenetic basis. However, most environmentally acquired epigenetic marks, even if introduced into the germline, do not typically propagate to consecutive generations. Nevertheless, there is evidence that some epigenetic marks can be passed from one to next

generation. Also, the original interpretation of epigenetic information embedded in DNA methylation and chromatin modifications has been expanded to include other molecules, including RNA and prions, which may have a higher probability to be passed along generations via the gametes.

DNA methylation and chromatin modifications as possible carriers of transgenerational information. DNA methylation involves the addition of a methyl group to cytosines, typically in a CG, but also in CA, CT, or CC context. While the majority of CG sites are methylated, there are areas with reduced or no methylation, such as CG islands (CGIs). Also, regions can have tissue-specific methylation differences. Maintenance methylation in principle provides a mechanism ensuring that methylation is not lost during mitotic cell divisions. The DNA is associated with histones that have their own heritable secondary modifications (Turner, 2007). DNA methylation, in conjunction with DNA bound histones, controls the accessibility of transcription factors and cofactors to the DNA; thus modulating gene expression (Bird, 2002).

Reprogramming in the germline and early embryo. Although not all, most information embedded in DNA methylation and histone modification is typically not passed to the next generation, because of reprogramming. In mammals, two major waves of reprogramming occur; one in the primordial germ cells (PGCs, the progenitors of oocytes and sperm) and another in the early embryo. Reprogramming is best understood in the mouse and involves the genome-wide erasure and subsequent establishment of DNA methylation and histone modification profiles, at each of the two reprogramming events (Rose *et al*, 2013).

DNA demethylation occurs between embryonic (E) day 8 and 12.5 in PGCs, while they migrate toward the genital ridge. Demethylation occurs simultaneously in both males and females and is accompanied by a reduction in the repressive mark H3K9 dimethylation (me2) and an increase in another repressive mark H3K27 trimethylation (me3). The reciprocal relationship between these modifications may facilitate the maintenance of repression patterns during epigenetic reprogramming. Demethylation seems to occur via conversion to 5-hydroxymethylcytosine, driven by high levels of tet methylcytosine dioxygenase 1 and 2 (TET 1 and 2), and completed by replication-coupled dilution (Seisenberger *et al*, 2012; Hackett *et al*, 2013). By E13.5, replication of germs cells and reprogramming are completed. Female germ cells enter meiosis I, but are halted at prophase I, persisting as primary oocytes until menarche, while male germ cells undergo mitotic arrest and stay at this stage until puberty. From puberty, oocytes and sperm are produced through meiosis. Global methylation in the sperm and oocyte is 90% and 40%, respectively (Kobayashi *et al*, 2012). While methylation in sperm is mostly limited to repetitive elements and intergenic sequences with relatively few methylated CGIs, the oocyte has more than a thousand

methylated CGIs (Borgel *et al*, 2010; Smallwood *et al*, 2011; Kobayashi *et al*, 2012; Smith *et al*, 2012).

The second phase of reprogramming occurs in the early embryo. Active demethylation in the zygote, involving extensive hydroxymethylation (paternal allele), is followed by the passive loss of methylation (both paternal and maternal alleles) during early cell divisions. Subsequently, remethylation takes place during the transition from blastocyst to epiblast (Smith *et al*, 2012).

Escape from erasure. Although reprogramming is genome wide and extensive during female PGC development (Seisenberger *et al*, 2012; Seisenberger *et al*, 2013), 4730 loci were shown to escape demethylation, predominantly repeat sequences (>95%), including IAP elements (intra-cisternal A particle) of the family of endogenous retrovirus (ERV) elements (Hackett *et al*, 2013). In addition to repeat sequences, 11 single loci at methylated CGIs were found to escape reprogramming in female E13.5 PGCs (Hackett *et al*, 2013). Another study found a subset of ERV1 elements and 23 single loci, of which 19 are not flanked by IAPs and have no apparent repeat sequences, to resist demethylation, in both male and female E13.5 PGCs (Guibert *et al*, 2012). Importantly, erasure-resistant single loci typically also retain their methylation in the early embryo (Guibert *et al*, 2012; Seisenberger *et al*, 2012). Although these loci are potential candidates for transgenerational epigenetic inheritance, the environmental malleability of their methylation or chromatin structure is not known.

Although sequences that are resistant to germline erasure typically also escape reprogramming in the early embryo (providing a possible substrate for transgenerational epigenetic inheritance), there are additional methylated sequences in the zygote that survive reprogramming in the early embryo. These include imprinted sequences and most of the oocyte and a few sperm-specific methylated CGIs (Smith *et al*, 2012). Another study identified ∼100 non-repeat and non-imprinted gene promoters whose methylation is not erased during early development (Borgel *et al*, 2010). These single locus sequences could mediate the gametic transmission of traits to the F2 generation, via the maternal line, because environmental exposure of the pregnant female could affect not only the F1 embryo, but also the F2, already reprogrammed, primary oocytes. As these methylation marks are typically erased in the germline, the traits would not be transmitted to the F3 generation. This 'F2-specific' epigenetic mechanism may explain the observation that some phenotypes can skip the F1 generation or can be stronger in the F2 than in the F1 generation, seen in both humans and in animal models (Wehmer *et al*, 1970; Weissman and Jensen, 2002).

Some nucleosomes may also escape removal during reprogramming, even in sperm, in which most of the histones are replaced by protamines. These reprogramming-resistant sperm nucleosomes are enriched in H2K27me3 and it has been suggested that this histone modification may represent a paternally transmitted transgenerational signal (Hammoud *et al*, 2009; Brykczynska *et al*, 2010).

Evidence for transgenerational epigenetic inheritance. Currently, non-genetic inheritance of traits has not been linked directly to gametic epigenetic marks that persist to the embryo and somatic tissues and then appear in the next generation germ cells. However, a number of reports described gamete-mediated transmission of behavior accompanied by epigenetic marks in the gametes and brain of the progeny. In a series of now classical papers, it was shown that transcription at the agouti-viable yellow and axin-fused loci is regulated by the methylation status of embedded IAP elements, which drives the phenotypes; a range of coat colors from yellow (low methylation/high expression) to brown (high methylation/low expression) for agouti and kinky tail (low methylation/high expression) for axin fused (Morgan *et al*, 1999; Rakyan and Whitelaw, 2003; Rakyan *et al*, 2003; Blewitt *et al*, 2006). The methylation status of the IAP at the parental agouti-viable yellow and axin-fused loci is recapitulated in the somatic cells of the offspring (in the maternal and in both the paternal and maternal offspring, respectively). As IAP elements are typically resistant to germline and early embryonic erasure, it was expected that methylation at the agouti-viable yellow and axin-fused alleles survive reprogramming, demonstrating that methylated DNA can be the carrier of intergenerational information. However, the IAP elements at both loci were found to be completely demethylated in blastocysts, derived from mothers carrying the methylated epialleles, indicating that methylation is not the epigenetic mark that carries the information across generations (Blewitt *et al*, 2006; Fernandez-Gonzalez *et al*, 2010). Then, it has been hypothesized that histone marks represent the epigenetic memory that survive erasure at these loci, because the penetrant (kinky tail) and silent axin-fused epialleles were associated with differences in histone H3K4 me2 and H3K9 ac marks at the blastocyst stage (Fernandez-Gonzalez *et al*, 2010).

Importantly, the agouti-viable yellow and axin-fused loci are environmentally malleable, because their methylation can be increased and the number of yellow and kinky tailed offspring decreased by maternal diet rich in methyl donors (folic acid, betaine, vitamin B12) (Wolff *et al*, 1998; Waterland and Jirtle, 2003; Cropley *et al*, 2006; Waterland *et al*, 2006). However, whether these diet-induced methylation changes are transgenerational is debated, as one report found no evidence for transmission (Waterland *et al*, 2007) while another described the appearance of the maternal phenotype in the next generation (Cropley *et al*, 2006).

In a recent paper, transmission of paternal sensitivity (startle response) to a specific odor, established by odor–shock pairing before mating, was reported through the paternal line to at least two consecutive generations (Dias and Ressler, 2013). Reduced DNA methylation was seen at the stimulus-associated odor receptor gene (*Olfr151*) in the sperm of both the exposed father and his naïve F1 offspring. Although these experiments do not establish a causal

relationship between hypomethylation at *Olfr151* in sperm and the non-genetic inheritance of odor sensitivity, the phenotype was transmitted even following *in vitro* fertilization and cross-fostering, indicating that some information in the gametes are linked to the non-genetic inheritance of the behavioral phenotype.

A similar situation is exemplified by maternal separation that leads to depressive-like behaviors in the adult F1 (stressed) offspring, manifested as deficits in novelty response, risk assessment, and social behaviors (Franklin *et al*, 2010; Weiss *et al*, 2011). These behaviors are transmitted through both the male and female lines up to the third generation and are accompanied by DNA methylation changes at stress-related gene promoters in F1 sperm and F2 brains. Likewise, paternal cocaine administration results in reduced sensitivity to the drug in the male offspring that is accompanied by altered histone 3 acetylation at the brain-derived neurotrophic factor (BDNF) gene in the sperm of the exposed father and in the brain of the offspring. In addition, expression of BDNF mRNA and protein was increased in the medial prefrontal cortex of the offspring (Vassoler *et al*, 2013). As inhibition of BDNF signaling reversed the phenotype, the epigenetic change in sperm and its presence in progeny brain may be directly related to the transmission of behavioral phenotype. Finally, in a model of fetal alcohol syndrome, it was shown that offspring, exposed during gestation to alcohol, exhibit a deficit in proopiomelanocortin (POMC)-related functions (for example stress hormone expression), accompanied by increased CpG methylation in the *POMC* promoter (Govorko *et al*, 2012) and that suppression of DNA methylation can normalize the functional abnormalities. Fetal alcohol-induced DNA methylation and functional defects persisted in the F2 and F3 male, but not in female, and promoter hypermethylation was detected in sperm of fetal alcohol-exposed F1 offspring that was transmitted through F3 generation via male germline. However, the ultimate proof to establish transgenerational epigenetic inheritance in all of these experiments is to rescue the transmitted phenotype by normalizing the environmentally induced epigenetic change in sperm. Although this approach is challenging, it is now feasible with recently developed site-directed genome-editing technologies.

RNA as carrier of transgenerational information. RNAs represent a new group of 'substrates' that could carry information across multiple generations via the gametes. The first data suggesting an RNA-mediated transgenerational effect in mammals was the identification of abnormal transcripts in sperm, produced from a structurally modified *Kit* parental allele (encoding a tyrosine kinase receptor), that resulted in a 'white tail' phenotype in the genetically WT offspring across multiple generations (Rassoulzadegan *et al*, 2006). Microinjection of total parental RNA into fertilized eggs reproduced the phenotype indicating that RNA was the carrier of phenotypic information. As this 'paramutation'-like phenomenon in the mouse involves

engineered mutations in the parental allele, the significance of this finding could be limited. However, two microRNAs with sequences partially complementary to that of Kit RNA (miR-221 and -222) induced the paramutated state (Rassoulzadegan *et al*, 2006). Moreover, a screen for paramutagenic miRNAs identified miR-1 and miR-124 causing paramutation-like effects on *Cdk9* or *Sox9* manifested as transgenerational cardiac hypertrophy and accelerated embryonic growth, respectively (Wagner *et al*, 2008; Grandjean *et al*, 2009; Cuzin and Rassoulzadegan, 2010). Interestingly, the mir-124-related phenotype was related to the establishment of a distinct, heritable chromatin structure in the promoter region of *Sox9* (Grandjean *et al*, 2009). Overall, these data indicate that sperm, and presumably oocyte, miRNAs can be passed to the embryo resulting in gene expression (Bartel, 2009) and phenotypic changes in the developing and adult progeny.

The possible role of RNAs in transmitting behavioral phenotypes is just beginning to be explored. One example is related to the non-genetic inheritance of increased stress responsiveness induced by maternal gestational stress in two consecutive generations of males (Mueller and Bale, 2008). The transmission of the stress phenotype was accompanied by reductions in miR-322, miR-574, and miR-873 in the brain and increased expression of their common target, β-glycan (TGFβr3) (Morgan and Bale, 2011). In a similar study, naïve males, rather than pregnant females, were exposed to chronic stress during puberty or in adulthood, which led to significantly reduced HPA stress axis responsivity in their offspring (Rodgers *et al*, 2013). The sperm miRNA content of stressed males was significantly altered with a total of nine miRNA increased. However, it is currently not known if the stress-induced miRNA profile is directly responsible for the reprogramming of the HPA axis. Another group of small RNAs, potentially relevant to transgenerational epigenetic inheritance, is represented by germ cell PIWI-interacting RNAs (piRNAs) (Luteijn and Ketting, 2013), but their involvement in non-genetic transmission of behavior is not yet known.

Future Research Directions

In conclusion, beyond the fundamental scientific interest in understanding how behavioral variability can propagate across generations via a non-genetic route, non-genetic inheritance has translational significance, because even a short period of parental exposure can increase the risk for neuropsychiatric disease in consecutive generations. Information transfer between generations can occur through multiple mechanisms. Although the most conceivable way of non-genetic inheritance is via the gametes, only a limited number of studies show unequivocally that gametes are the carriers of this information. Indeed, experiments employing embryo transfer or *in vitro* fertilization are required to prove that the phenotype is transmitted via the gametes (Figure 2). Unfortunately, very few studies actually employed these techniques and therefore, third and fourth

generation traits described in the literature are not necessarily gamete mediated. The epigenome is reprogrammed through gametogenesis and in the early embryo, and only few traits are likely to be truly epigenetic in nature, therefore warranting a cautious interpretation. In principle, there are two requirements for a molecule or molecular modification to carry epigenetic information across several generations. First, it has to be associated with the parental gametes (whether in the nucleus, cytoplasm or even structures associated with the gametes) and be stable enough to survive until fertilization. Second, the molecule/molecular modification or its signal has to be propagated through embryogenesis and then through gametogenesis and reprogramming. A number of molecules may be suitable carriers of epigenetic inheritance, including secondary DNA and histone modifications and small RNAs. Non-gametic inheritance is equally complicated mechanistically, as multiple parental–offspring pathways exist with very different molecular and cellular basis. These mechanisms include social/behavioral, immunological, and microbial interactions. Similar to the gametic non-genetic inheritance, rigorous standards are required to establish non-gametic inheritance because the possible confounds of spurious genetic effects. For example, toxin-induced mutations in the parents or spurious parental mutations, that are transmitted genetically, could underlie the transmission of a trait. To exclude these genetic confounds, the best practice is to transfer early embryos or cross-foster newborns from unaffected parents to mothers/parents exposed to the environmental factor, depending on whether the effect is pre- or postnatal, respectively. It is expected that both non-gametic and gametic non-genetic inheritance contribute to the high incidence of psychiatric disorders. Therefore, one may also expect that the two forms of non-genetic inheritance carry symptom-specific information simultaneously that, together with genetic traits, will explain the complex and variable phenotypes of psychiatric disorders. The varying mechanisms of inheritance may call for different preventive measures and could influence the strategies employed to prevent and possibly mitigate disease symptoms.

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GLOSSARY

(some definitions may differ from those used in other reviews)

Inheritance. The process by which phenotypes (traits or characters) are passed from parents to offspring.

Non-genetic inheritance. Inheritance is typically associated with genetic or Mendelian inheritance. However, traits can be passed from parents to offspring via mechanisms that do not involve the DNA sequence. Therefore, inheritance is a broader term that includes non-genetic mechanisms of inheritance.

Gamete. A mature male or female germ cell that fuses with a gamete of the opposite sex during fertilization.

Epigenetic marks/signatures. In addition to the DNA sequence, gene expression information is embedded in additional layers on the DNA, termed epigenetic marks/signatures. Cells acquire epigenetic marks during their development. Epigenetic marks include chemical modifications of the DNA and secondary modifications in histones. Small RNAs are also often viewed as epigenetic signals/signatures because, *a priori*, any mechanism that provides regulatory information to a genome could be considered epigenetic. In contrast to the DNA sequence, epigenetic marks are malleable by the environment and their changes by the environment can significantly alter the cellular phenotype. In fact, it is believed that adaptation to the environment, learning, and many environmentally related processes involve changes in the epigenetic state of alleles.

Epialleles. Alleles those are identical in sequence, but different in epigenetic modifications.

Non-genetic inheritance via the gametes. A form of non-genetic inheritance that is mediated by the gametes without the involvement of DNA sequence. As epigenetic marks in the gametes are believed to carry the information from parents to offspring, this mechanism is also referred to as 'transgenerational epigenetic inheritance'. However, any biological compound within or associated with the gametes, excluding the DNA sequence, may serve as a carrier of non-genetic information.

Gestational and early postnatal non-genetic inheritance. A process that involves the transfer of traits from the mother to offspring during gestation, or from the parents to the offspring during the early postnatal period, which typically spans from birth to weaning. It is also called 'maternal/parental programming'. Mother/father information transfer can occur via the immune system, metabolites, and the microbiome, but also through social/behavioral interaction.

Multigenerational non-genetic effects. The acquisition and propagation of novel phenotypes across multiple generations that are not genetically based.

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