Feature Review

CellPress

# Epigenetic mechanisms underlying learning and the inheritance of learned behaviors

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**Gene expression and regulation is an important sculptor of the behavior of organisms. Epigenetic mechanisms regulate gene expression not by altering the genetic alphabet but rather by the addition of chemical modifications to proteins associated with the alphabet or of methyl marks to the alphabet itself. Being dynamic, epigenetic mechanisms of gene regulation serve as an important bridge between environmental stimuli and genotype. In this review, we outline epigenetic mechanisms by which gene expression is regulated in animals and humans. Using fear learning as a framework, we then delineate how such mechanisms underlie learning and stress responsiveness. Finally, we discuss how epigenetic mechanisms might inform us about the transgenerational inheritance of behavioral traits that are being increasingly reported.**

## Bridging the gap between genes and the environment

The dynamic regulation of gene expression in response to environmental stimuli is vitally important for complex organisms to develop, adapt, and survive in multifaceted environmental conditions. The concept of epigenetic regulation (Box 1; Figure 1) may provide the framework for a mechanistic understanding of the mutual interaction of the genetic blueprint with changing environmental conditions. Here, the environment can lead to long-lasting modifications in genome organization and gene expression as a function of – but without changing – the underlying DNA sequence. In particular, the field of neuroepigenetics has gained much attention in the past decade providing exciting insights in the response of the brain to environmental cues, consequently regulating behavior but also the pathogenesis of mental disorders. Fear learning provides a framework within which to study how environmental cues leave their imprint on the nervous system. Here, we will focus on the epigenetic basis of fear memory learning and the transgenerational inheritance of learned sensitivities to environmental cues as a consequence of ancestral experiences.

## Epigenetic modification and the regulation of learning and memory

### *Fear learning*

Over the past decade, much effort has begun to examine the role of epigenetic mechanisms in the establishment of psychiatric disorders. Pavlovian fear conditioning, alternatively referred to as threat conditioning, has been proposed as a model to examine the epigenetic mechanisms that underlie the initial formation of an aversive fear memory [1]. Pavlovian fear conditioning serves as an excellent paradigm with which to access the cellular, molecular, and epigenetic mechanisms that underlie the initial formation of long-lasting memories and behavioral adaptations in adult rodents. It is worth noting that much work has also revealed critical roles for epigenetic regulation of spatial learning and memory, however, as no evidence for ancestral inheritance of these memories has yet to emerge, we have focused our discussion on animal models of learning and memory that have some suggestion of ancestral inheritance.

### *Histone regulation and fear memory formation*

Evidence for the emergence of neuroepigenetics was first revealed with the demonstration that contextual fear conditioning resulted in an increase in histone H3 acetylation within the hippocampus [2], suggesting for the first time a role for chromatin modifications in the formation of an aversive memory. In support of a role for chromatin modifications in memory formation, many labs have since demonstrated that inhibition of histone acetyltransferase (HAT) activity impairs training-related changes in histone acetylation and in concert impairs long-term fear memory formation in a variety of learning and memory tasks using transgenic mouse models or pharmacological agents [3–10]. Correspondingly, inhibition of histone deacetylase (HDAC) activity has been found to not only enhance training-related changes in histone acetylation but also enhances long-term memory using a variety of behavioral paradigms including contextual, auditory, and spatial memory tasks [11–16], fear memory reconsolidation [17],

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0166-2236/
 http://dx.doi.org/10.1016/j.tins.2014.12.003

**Box 1. A brief introduction to epigenetic modification**

In this review, we broadly define epigenetics to imply the mechanisms influencing gene expression without changing the underlying genetic sequence and we focus on histone modifications, DNA methylation, and noncoding RNAs (Figure 1). While a detailed description of each of these modes of epigenetic regulation of gene expression is beyond the scope of this review (Figure 1), we briefly discuss salient features of these modes and point readers to more in-depth literature [1,2] for a more comprehensive discussion of their nuances. Although depicted separately below, epigenetic modifications form a complex interactive network with cooperating effects on transcriptional regulation in a spatial and temporal manner.

**Post-translational modification of histone proteins**: DNA is highly condensed into chromatin, allowing for it to be condensed into chromosomes in the nuclei. Chromatin consists of DNA wrapped around histone proteins. These histone proteins can be chemically modified at specific residues by a plethora of post-translational modifications including acetylation, methylation, phosphorylation, and sumoylation predominantly at their N-terminal tails. This unique chemical signature influences the overall chromatin structure and binding of DNA binding proteins, and allows for the loosening or tightening of the chromatin around particular genetic loci leading to the facilitation or suppression of gene transcription [3].

**Cytosine DNA methylation**: the covalent modification of cytosine residues mainly in the context of CpG dinucleotides can influence gene expression through enhancing or decreasing the binding of transcriptional regulatory proteins with decreased [4] but also increased transcription reported. The classical view of increased DNA methylation in promoter/first exon regions leading to reduced gene expression has been broadened by findings showing that increased DNA methylation in the gene body can actually lead to an increased transcription, thus implicating a bidirectional regulation of transcription depending on the location of the DNA mark. More recent work points toward distinct methyl-modifications such as the 5-hydroxy methylation implicated in DNA de-methylation but also affecting gene transcription in particular in neuronal tissue [5,6].

**Noncoding RNA**: transcriptional regulation and chromatin organization through small ($<$ 200 nt) or long ($>$ 200 nt) nonprotein coding RNAs include, for example, miRNA and piRNA that have distinct mechanisms by which they regulate gene expression [7]. miRNA are thought to exert their effects via target mRNA degradation or translational repression, while piRNA do so via inhibition of RNA polymerase II transcription. More recently, long noncoding RNAs have also been implicated in transcriptional and post-transcriptional regulation as well as in interplay with other epigenetic mechanisms [11]. Expression of these noncoding RNAs in somatic cells, as well as in the germline, have made them attractive candidates to mediate transgenerational epigenetic inheritance [8–10].

and has been found to expedite the extinction of fear memory [18–20]. The common finding of memory enhancement via HDAC inhibitor treatment has led many researchers to discuss the promise of HDAC inhibitors in the treatment of memory disorders and in conjunction with exposure-based therapy for phobias and fear-based memory disorders such as post-traumatic stress disorder (PTSD) [21]. While studies employing pharmacological inhibitors of HDAC activity are useful, these compounds may also affect other non-histone protein targets. Thus formal conclusions of the role of HDACs in memory should be made cautiously in light of their off target and complex effects. Additional studies have begun to also highlight a role for histone phosphorylation in initial memory formation [22,23] as well as delineating the role of histone methylation in memory formation [24,25]. Unlike histone acetylation, which facilitates transcription, the consequences of histone methylation vary as a function of which lysine reside is methylated (i.e., H3K9me or H3K4me) and the degree to which the residue is methylated (mono-, di-, or tri-). The methylation status of H3K9me2, a mark which is generally considered repressive of transcription, and H3K4me3, a mark permissive to transcription, have been recently demonstrated to be regulated in the hippocampus with contextual fear memory formation. In agreement with H3K4me3 being permissive for transcription, additional experiments determined that there was an increased occupancy of H3K4me3 within the *BDNF* (brain-derived neurotrophic factor) and *zif268* (zinc finger protein) promoters, both genes that are critical for fear memory formation. Interestingly, this study also determined that the classical repressive mark H3K9me2 was increased as a consequence of context exposure only and contextual fear conditioning; it remains unclear why both repressive and permissive histone marks are upregulated as a consequence of fear conditioning. However, it is also possible that each mark plays a different role in regulating gene transcription critical for memory formation. Whereas the increase in H3K4me3 may be associated with permitting the transcription of memory supporting genes, the increase in H3K9me2 may mediate the inhibition of memory suppressing genes, a balance which would be in favor of long-term memory formation [26]. Another possibility is that the epigenetic marks are being utilized by different cell types of opposing function, but that with tissue punches, as done in almost all of the studies to date, signals from a large variety of cells are indistinguishable.

Interestingly, we have recently demonstrated that auditory fear conditioning was associated with a reduction of H3K9me2-occupancy at the promoter region of *homer1a*, a gene which appears to be critical for long-term memory formation, in the amygdala [27]. The reduction in H3K9me2 occupancy at the *homer1a* promoter was found to be associated with an increase in *homer1a* mRNA and suggests that training-related reductions in H3K9me2 are critical for mediating the transcription of genes necessary for fear memory formation. Although this study did not examine the global regulation of H3K9me2 in the amygdala with auditory fear conditioning, it remains possible that H3K9me2 may be decreased within specific gene promoters while it remains intact or upregulated in others, again to facilitate the notion of critical balance in memory suppressing or promoting gene transcription. Additionally, it is possible that there may be differential chromatin modifications that are engaged in the amygdala and hippocampus and are determined by the behavioral task at hand, that is, contextual or auditory fear conditioning. While these studies have demonstrated that histone modifications are dynamically regulated at the time of memory formation and correlate with the expression level of genes, only until recently did the technology exist, in the form of Transcription Activator-Like Effector (TALE) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems, to enable targeted perturbations of histone modifications and DNA methylation at specific gene loci. To our knowledge, this technology has not yet

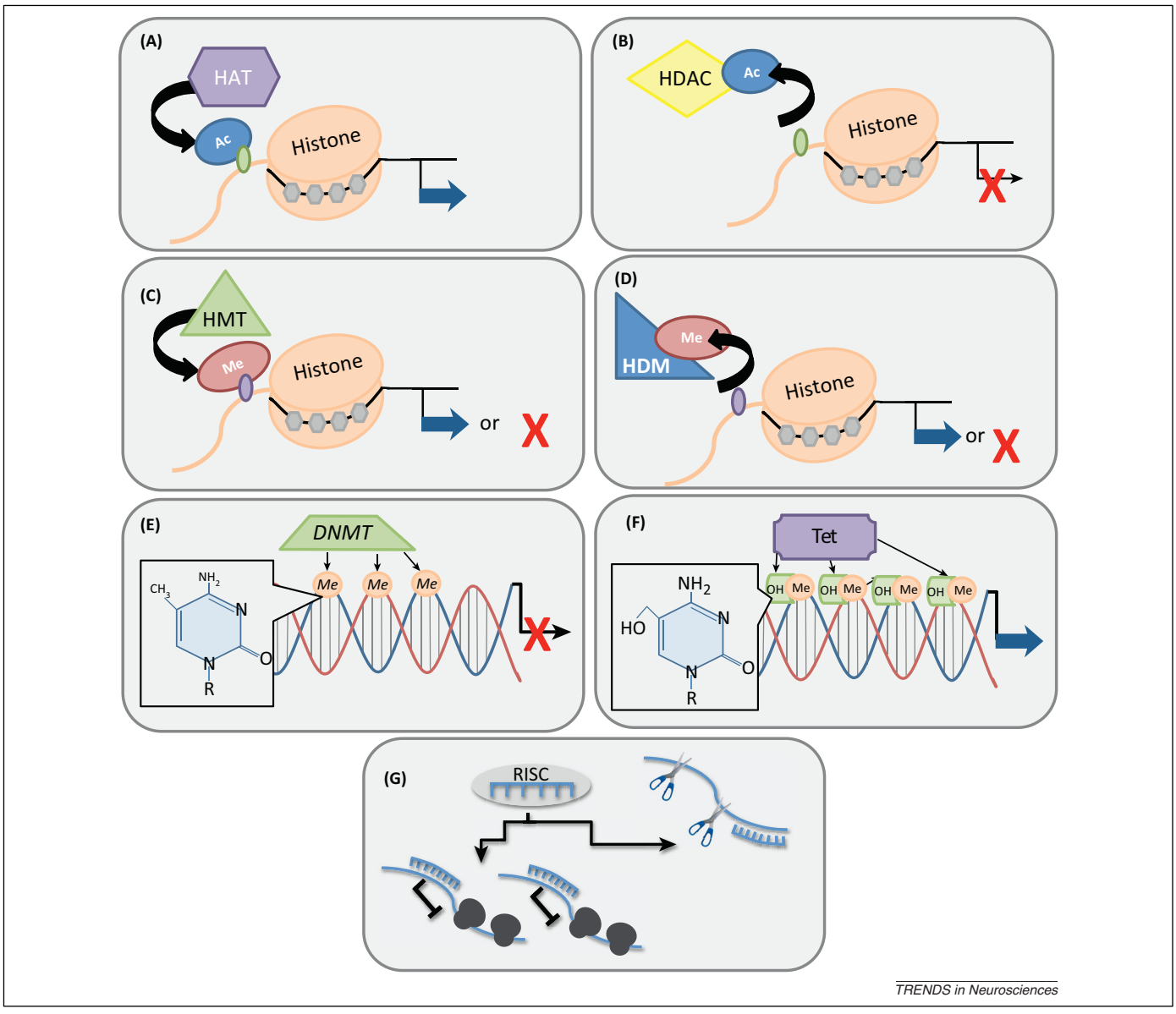


**Figure 1**. Overview of epigenetic regulatory mechanisms. This schematic diagram demonstrates the primary known functions of the different enzymes referred to within the review. **(A)** Histone acetyltransferases (HATs) add acetyl groups to lysine residues on histone tails, and are generally associated with relaxing wound DNA and promoting transcription. **(B)** Histone deacetylases (HDACs) remove those acetyl groups, and inhibit transcription. **(C,D)** Histone methylation is mediated by histone methyltransferases (HMTs), which add methyl groups to lysine residues on histone tails, and this process is reversed by histone demethylases (HDMs). The impact of histone methylation on transcription largely depends on the lysines and state of methylation (mono-, di-, tri). **(E)** DNA methyltransferases (DNMTs) add methyl groups to the cytosines of CpG islands, resulting in a 5-methylcytosine (5mC) state, and are generally associated with DNA silencing. **(F)** Active demethylation has recently been associated with ten-eleven translocation methylcytosine dioxygenase (Tet) protein-mediated hydroxylation of 5mC, resulting in 5-hydroxymethylcytosine (5hmC), and has been found to facilitate transcription. **(G)** A schematic diagram of miRNA-mediated inhibition of gene translation and mRNA degradation via the RNAi Silencing Complex (RISC) as examples for epigenetic regulation by small noncoding RNAs.

been employed to examine how these perturbations at specific gene loci impact learning, memory, stress, or their ancestral inheritance.

### *DNA methylation and memory*

A wealth of studies have noted a critical role for covalent DNA modifications in memory formation. Alterations in DNA methylation status at gene loci, long considered to be a static process in the initial formation of a contextual fear memory, have been demonstrated through the use of DNA methyltransferase (DNMT) inhibitors [13,28–30]. While inhibition of DNMT activity has been found to impair both contextual and auditory fear memory formation [11,29,31], the mechanism behind this effect has not yet been well elucidated. Recent work has also revealed a role for active DNA demethylation in memory formation by demonstrating a critical role for the ten-eleven translocation methylcytosine dioxygenase 1 (Tet1) protein, which mediates the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) to promote transcription [32]. Tet1 knockout mice have been found to have impaired contextual fear and spatial memory formation [33,34], suggesting that Tet1 is critical for long-term memory formation. Upon closer examination, the memory deficits observed with Tet1 knockout were associated with training-related deficits in many immediate-early genes known to be critical

for memory formation; suggesting that the memory deficits result from impaired Tet1-mediated transcription. While the mechanisms of active and reversible DNA demethylation in neurons in response to behavioral experience have yet to well investigated, the plethora of studies demonstrating deficits with DNMT inhibition, and more recently Tet1, warrant further exploration.

### *Noncoding RNAs and learning and memory*

A role for microRNA (miRNA)-mediated regulation of gene transcription has recently emerged within the field of learning and memory. A role for miRNAs in the initial formation of fear memories was observed using Dicer knockout mice, the enzyme critical for producing mature miRNAs via mediating the cleavage of RNAs into miRNAs, which displayed enhanced performance on the Morris water maze, and in cued and context fear conditioning tasks [35]. More recently, bioinformatics approaches have contributed to the identification of novel miRNA targets such as miR-34a and miR-182, which have both been found to be actively regulated in the amygdala at the time of fear memory formation [36,37], where miR-34a was increased and miR-182 expression was decreased after conditioning. These studies have further supported the role of miRNAs by demonstrating that overexpression of miR-182 and inhibition of miR-34a in the amygdala both impair fear memory consolidation. Interestingly, novel mechanisms have been put forth to propose a mechanism for these effects on memory. Whereas miR-34a has been found to impair the regulation of Notch signaling pathways at the time of fear conditioning to promote memory formation, miR-182 has been found to impair actin-related genes known to be critical for memory formation. Although it is outside the scope of this review, it is worth mentioning that miRNAs have also been found to be involved in the initial formation of fear extinction memory [38]. These findings taken together signify the active regulation of miRNAs with initial fear memory formation in the amygdala and highlight the intricacies of miRNA-mediated gene regulation.

Overall, there are many ways in which epigenetic modifications, from histone regulation and DNA methylation to noncoding RNA effects, can all lead to differential gene expression, downstream of a cascade of cellular transduction events (Figure 2). The discussed work demonstrating both the regulation and necessity of histone modifications in learning and memory also suggests that a 'histone code' consisting of post-translational modifications and DNA methylation are recruited at the time of memory formation by enzymatic process (HATs, HDACs, etc.) to establish a

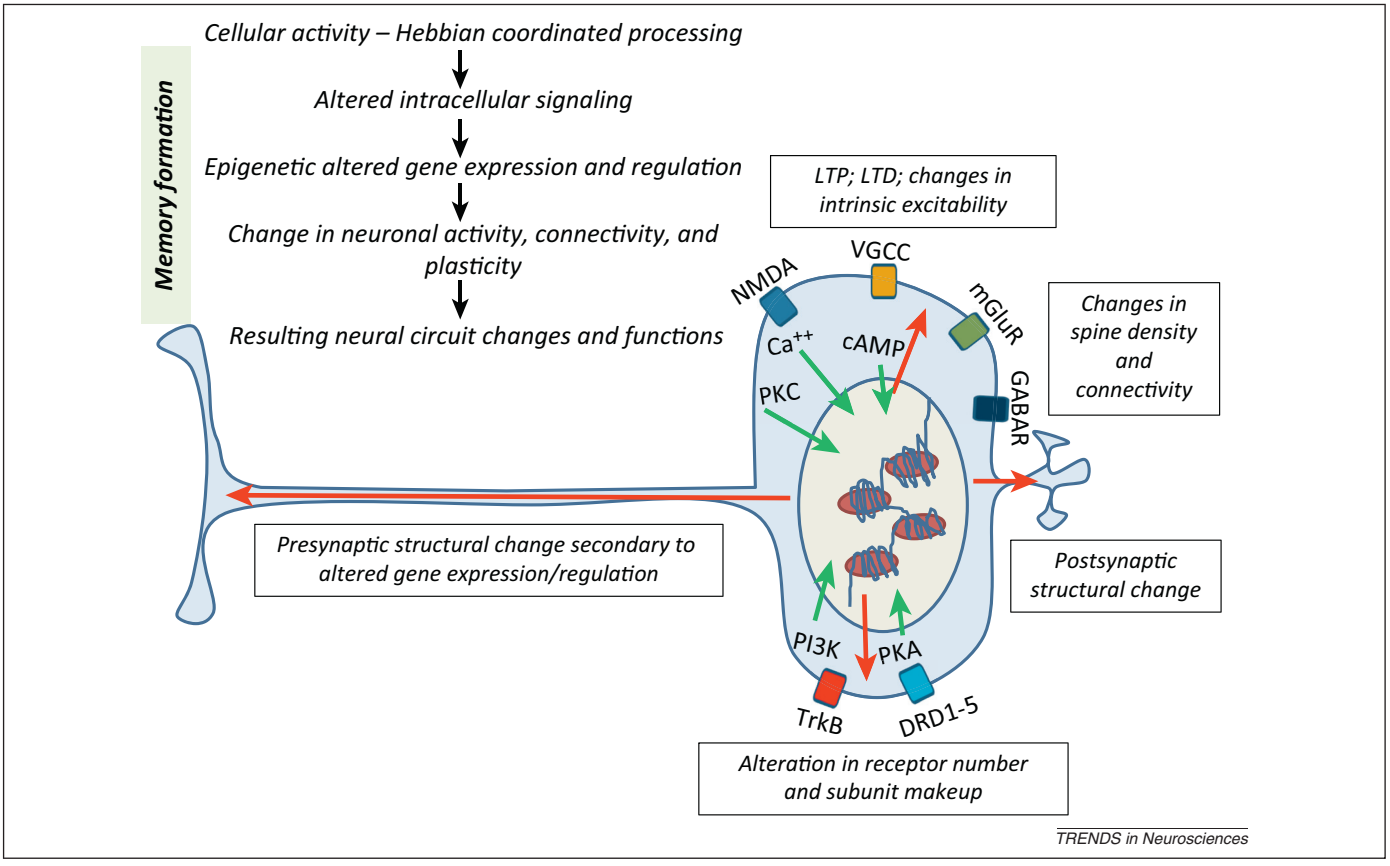


**Figure 2**. How epigenetic regulation of neuronal gene expression may alter neuronal plasticity and activity, resulting in memory formation. This schematic diagram illustrates various mechanisms, supported by published data, by which coordinated cellular activity leads to altered intracellular signaling, with resultant epigenetic alterations at the levels of noncoding RNA, histone regulation, and DNA methylation. Together such changes alter regulation of gene expression, resulting in postsynaptic changes at the levels of spine density, receptor sensitivity, and intrinsic excitability, etc., as well as providing the substrates for altered presynaptic structural and functional change. The changing of the neuronal state at the level of epigenetic gene regulation interacts with local determinants related to synaptic connectivity and circuit activity, which together alter neurocircuitry dynamics underlying memory formation. Abbreviations: DRD1-5, dopamine receptor D1-5; GABAR, GABA receptor; LTD, long-term depression; LTP, long-term potentiation; mGluR, metabotropic glutamate receptor; PI3K, phosphatidylinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; TrkB, neurotrophic tyrosine kinase receptor type 2; VGCC, voltage-gated calcium channel.

gene-specific code to dictate whether a gene is actively transcribed or repressed at the time of learning [39], to ultimately influence the formation of memory.

### *Epigenetic modification related to stress and addiction*

Animal models examining the mechanisms that underlie the consequences of stress have also revealed an integral role for epigenetic mechanisms. Recently, exposure to acute restraint stress has been found to result in increased levels of histone H3K9me3, a mark associated with heterochromatin dynamics and transcriptional inhibition, and an increase in Suv39h2, the histone methyltransferase (HMT) that mediates the methylation status of H3K9 [40,41]. The increase in the repressive H3K9me3 mark with stress history may suggest a novel theory that the dysregulation associated with stress and anxiety may be attributable to failure to engage or actively transcribe genes that may buffer the negative effects of stress and facilitate resiliency, an interesting hypothesis that remains not yet well tested.

Additionally, as much work has noted that the glucocorticoid receptor (GR) is integral in the stress response system due to its activation by the hypothalamic–pituitary–adrenal axis (HPA) axis [42], recent studies have turned to examine how stress exposure may epigenetically alter GR signaling (reviewed by McGowan *et al.* [43]). In particular, recent studies from both animal models and a human clinical population have examined the epigenetic regulation of *fkbp5* (FK506 binding protein 5), a gene that regulates GR receptor translocation and functions as part of a negative feedback loop to govern GR dynamics (Figure 3A). Animal studies have suggested that *fkbp5* may be critical for mediating coping responses and have demonstrated that *fbkp5* knockout mice have reduced HPA reactivity in response to stress [44,45]. Further, employing a chronic stress model, which involves chronic corticosterone exposure, *fkbp5* mRNA was found to be increased in the hippocampus, hypothalamus, and blood of mice [46]. Interestingly, this increase in fkbp5 mRNA was associated with a decrease in DNA methylation within *fkbp5* intronic regulatory elements and reduced DNMT1 expression [46]. This reduction in *fkbp5* methylation evident in blood samples was found to be associated with increased anxiety and correlated with glucocorticoid load [47]. These findings demonstrating decreased *fkbp5* methylation and increased *fkbp5* mRNA evident as a consequence of chronic corticosterone exposure illuminates the ability to correlate epigenetic modifications of *fkbp5* using a biomarkers-based approach with the behavioral consequences associated with a stress phenotype.

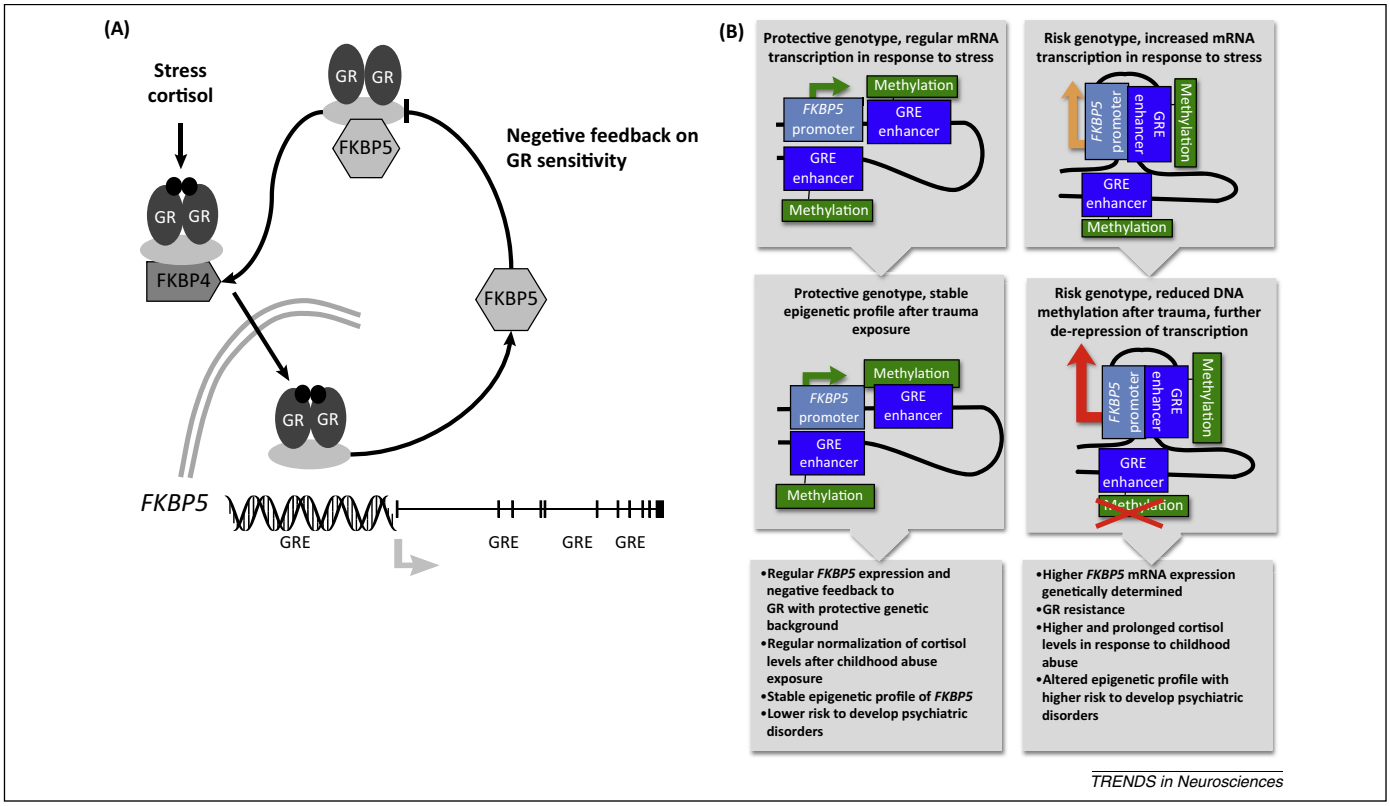


**Figure 3**. Epigenetics and stress responses: example of the *FKBP5* (FK506 binding protein 5) gene. *FKBP5* regulates the HPA axis and is epigenetically modified by childhood abuse in a genotype-dependent manner. **(A)** FKBP5 is a co-chaperone of the glucocorticoid receptor (GR), binding via heat-shock protein 90 (hsp90) and reducing its affinity for cortisol thus providing an ultra-short feedback loop to limit hypothalamic–pituitary–adrenal axis (HPA) activation. In response to cortisol binding, *FKBP5* is replaced by *FKBP4*, which facilitates the translocation of the GR complex into the nucleus where the GR binds to glucocorticoid response elements (GREs). Among other stress-responsive genes, *FKBP5* transcription and translation is increased via intronic response elements, which confers higher GR resistance, serving as an ultra-short negative feedback loop on GR sensitivity. **(B)** Epigenetic regulation of *FKBP5* in response to childhood abuse. The genetic predisposition in *FKBP5* determines the 3D organization of the *FKBP5* locus and the stress-dependent transcriptional activation of the gene with higher mRNA expression in risk allele carriers due to the increased interaction of distal GREs. In response to childhood abuse, carriers of the protective genotype maintain a stable epigenetic profile, whereas in risk allele carriers, trauma induces a demethylation in the GRE with further de-repression of *FKBP5* transcriptional activation. The resulting HPA axis deregulation contributes to the development of psychiatric disorders. Adapted from Klengel *et al.* [50].

In parallel to the murine studies mentioned above, *FKBP5* also serves as prominent model for gene by environment interaction studies (GxE) in humans [48,49], a layer of complexity that is not readily addressed in rodent studies so far. Human genetic studies largely failed to initially identify robust main effects for genetic signatures related to environmentally-influenced psychiatric phenotypes such as PTSD or major depression. The concept of GxE integrates both genetic variation between individuals and environmental exposure on the development of behavioral phenotypes. Among the investigated genes, single nucleotide polymorphisms in *FKBP5* have been shown to interact with exposure to childhood trauma on the development of PTSD. More recently, we showed an allele-dependent epigenetic mechanism underlying this GxE. Exposure to childhood trauma leads to a genotype-dependent chromatin conformation change and subsequent reduction in *FKBP5* methylation at glucocorticoid binding sites resulting in increased transcriptional activation and altered HPA axis activation in humans (Figure 3B). This epigenetic modification was restricted to individuals carrying a risk genotype emphasizing the decisive role of genetic variations in the epigenetic response to environmental factors [50].

Broadly speaking, learning implies processing salient environmental cues and consolidating that salience to memory such that future encounters of these cues are met with appropriate behavioral outcomes. In some cases, descendant generations are privy to environmental factors that their ancestral populations are exposed to. In this context, these descendant generations are not necessarily learning about ancestral environments but are certainly navigating their own worlds by taking into account features in their ancestors' environments. In the next section, we discuss some instances that suggest epigenetic mechanisms underlying this inter- and trans-generational influence on descendant biology (Box 2).

## Transgenerational inheritance

### *Inheritance of traits as a consequence of stressful ancestral experiences*

Animal models that have investigated the role of epigenetic mechanisms in the inheritance of behavioral traits across generations have typically done so by subjecting ancestral generations (F0) to some form of stress. The consequences of this ancestral experience are then queried in descendant generations.

*DNA methylation-based mechanisms of inheritance.* As described in the outset, histone regulation and DNA methylation are the most common mechanisms understood related to epigenetic regulation. The relevance of such histone modifications in sperm and consequently transgenerational inheritance is unclear especially in light of most histones being replaced by protamines. However, evidence does exist for certain histone modifications to be retained in sperm and therefore contribute to the inheritance of epigenetic marks that might have been accrued via the male germline [51]. The presence of histone modifying enzymes within oocytes following fertilization also present a testable mechanism by which maternal experiences could be epigenetically inherited [52]. Notably, germ cells differ remarkably from other cell types with respect to their epigenetic configuration as well as epigenetic changes occurring during their development, fertilization, and further embryonic development. As mentioned before, most histone proteins are replaced by protamines in sperm [53]; both maternal and paternal genomes undergo substantial DNA methylation changes during development, fertilization, and further embryonic development [54]. In addition, germ cells are subjected to imprinting [55] and X-inactivation including the usage of unique epigenetic modifiers [57].

In the realm of maternal behavior, the transmission of quality of maternal care has been well documented [58]. High quality maternal care experienced by female rat pups at postnatal time-points faithfully results in them

**Box 2. Social transmission versus biological inheritance of traits**

Environmental information registered by an ancestral generation might be passed down to descendants via two routes: social transmission and biological inheritance. Social transmission involves either a direct interaction between the ancestral and descendant generation or an indirect interaction via maternal rearing environments influencing descendant biology. A feature of social transfer of information is the reversibility of effects when either (i) such aforementioned interactions and rearing environments are manipulated via cross-fostering studies, or (ii) the effects are no longer observed in multiple descendant generations. By contrast, biological inheritance speaks to the idea that the gametes (sperm and eggs) are marked by the salient environmental event, and that these marks are then inherited by descendants. Biological inheritance implies that any effects (i) ought to be recapitulated when IVF is used to generate descendant generations, (ii) ought not to be reversed when the rearing environment is manipulated as is done in cross-fostering designs, and (iii) persist in multiple descendant generations that are far removed from the perturbation of the ancestral environment.

In making a case for biological inheritance, in addition to the three points listed above, attention must be paid to the timing of perturbation to the ancestral population and the sex of the exposed generation. In terms of timing, while pre-conceptional, *in utero*, and postnatal exposure to perturbations profoundly affect descendants, they do so at different levels. Pre-conceptional perturbations to the F0 generation affect the germ cells of that generation, which will generate the F1 generation. *In utero* and postnatal perturbations by contrast affect the F1 generation and the germ cells of that F1 generation, which will form the F2 generation. Therefore, to qualify as inheritance, the trait in question must be seen at least in the F2 generation (when perturbation is pre-conceptional) and in the F3 generations (when the perturbation occurs *in utero* and postnatally). When the maternal lineage is exposed to an environmental manipulation, consequent alterations in maternal behavior might be the contributor to any effects observed in the F1 generation. In this scenario, the persistence of effects after cross-fostering would make a case for inheritance. In the absence of such studies as might be observed in human examples, the persistence of effects into the F3 generation after the F2 generation has been raised in standard rearing conditions would also make a case for the inheritance of phenotypes. The gold standard for inheritance after perturbation to the paternal generation is the persistence of effects after IVF.

In summary, a case for transgenerational inheritance could be made after accounting for the ancestral experiences of maternal versus paternal lineages, the timing of these experiences, rearing environments, and the use of techniques such as IVF.

engaging in the same high quality care toward their own offspring. From a physiological perspective, epigenetic marking via DNA methylation of the GR in the hippocampus and the estrogen receptor in diverse brain regions has been shown to be involved in how the descendant generations experiencing high or low quality maternal care navigate their environments in adaptive or maladaptive manners, respectively [59–61]. The reversal of these effects by cross-fostering does distinguish this social transmission of behavior and physiological changes from an inheritance mode of information transfer, but the lessons gleaned from studies like these have informed us about the salience and nuance of ancestral experience.

More recent work that documents a transgenerational inheritance of ancestral experience utilizes maltreatment during the postnatal care given to F0 rats and demonstrates that the effects of this maltreatment can be observed in the F1 generation with cross-fostering unable to reverse these effects. For example, subjecting infant rats to a stressed dam resulted in abusive behavior directed to the infants and in an altered epigenetic signature around *BDNF* in the prefrontal cortex (PFC) [62,63]. This altered epigenetic signature consequently resulted in alterations in *BDNF* expression. Of note were the observations that offspring (F1 generation) of the maltreated female F0 also had an altered epigenetic landscape around *BDNF*. Cross-fostering of infants born to stressed females by normal females did not rescue changes in *BDNF* epigenetic regulation, emphasizing an independence between the postnatal rearing environment and the epigenetic mode of information inheritance. Another study exposed infant mice (F1) to chronic and unpredictable separation from the mother (F0) during the first two postnatal weeks [64]. This stressful perturbation resulted in depressive-like symptoms in the F1 generation in adulthood, and extended to the F2 generation despite normal adult F2 rearing conditions. In addition, methylation status was queried in the DNA of the F1 sperm and F2 brain. A correspondence was found in that changes to the methylation profile of genes in the F1 sperm was accompanied by altered epigenetic signatures at the same genes in the F2 brain. These data exemplify how ancestral experiences leave imprints on the behavior, physiology, and epigenome of the descendants.

Another example of the inheritance of behavioral traits comes from a study that utilized social defeat of a male mouse and testing descendant generations [65]. Subjecting a male mouse to social defeat prior to mating resulted in the F1 offspring exhibiting depressive- and anxiety-like behavior, as well as higher levels of baseline corticosterone. Most metrics queried were not inherited in offspring derived from sperm of socially defeated males via *in vitro* fertilization (IVF), leading the authors to conclude that the effects observed were not strictly inherited. It must, however, be pointed out that the effect on latency to immobility in the forced swim test did persist after IVF, which is suggestive of some nuanced inheritance that an as yet unpublished mechanism might explain.

*Noncoding RNA-based mechanisms of inheritance.* A recent study subjected male mice to chronic stress for 6 weeks prior to mating [66]. While no baseline behavioral deficits were noted in the descendant generation, a hyporesponsiveness of the stress pathway was observed in the F1 generation in response to restraint stress. Gene expression was dramatically altered in the brain of the F1 generation. In pointing toward a potential mechanism for such effects on the F1 nervous system, the authors assayed expression of miRNA in the F0 sperm and found the miRNA profile to be altered as a consequence of the chronic stress experienced. While no published data currently exist to suggest that this altered miRNA panel in the sperm of F0 stressed males is directly responsible for the gene expression changes in the F1 nervous system, it would not be a stretch to entertain this possibility. The same research group has shown that early gestational stress has far reaching consequences on stress physiology and sexual differentiation with an altered miRNA profile in the brain of the descendant F2 generation espoused to be at the heart of these effects [67]. An miRNA has been shown to be the conduit via which fur coloration is inherited across generations [68], and it would be peculiar if such miRNA-mediated inheritance did not also extend to behavioral and physiological traits.

Further, a recent study by Gapp *et al.* showed that small RNAs are involved in the transmission of environmentally induced traits to offspring generations [69]. Using a combined approach with maternal separation and maternal unpredictable stress (MSUS), the authors were able to show that the small RNA content, including both miRNAs and PIWI-interacting RNAs (piRNAs) in F1 offspring (the generation that is directly exposed to the stressed mother animal), is in fact altered. These animals exposed to a stressed mother animal also showed behavioral and metabolic alterations that were similarly found in the F2 generation (the generation that was non-exposed to the stressed mother animal). Notably, the non-exposed F2 generation did not show differences in sperm small RNA content, which contrasts with previous findings of a similar behavioral phenotype in the F3 generation. The authors speculated that other epigenetic mechanisms than small RNAs such as DNA methylation or histone/protamine modifications subsequently carry the signal on to the F3 generation. Excitingly, the authors also show direct evidence for the transmission of these phenotypes by injecting miRNAs identified previously in oocytes resembling the behavioral and metabolic phenotypes found by MSUS treatment.

### *Inheritance of traits as a consequence of cue-specific ancestral experiences*

While the studies cited above eloquently address possible mechanisms underlying the transgenerational inheritance of ancestral traits, the perturbations to the ancestral generation are of a broad nature. Questions should also be asked as to whether the salience of specific cues in the ancestral environment could be inherited. This would enable descendants to navigate these cues in their own environments in a relevant manner. Most evidence for this specificity of descendants' response to discrete cues in the ancestral environment comes from studies of *in utero* learning. It should then come as no surprise that some such examples make use of chemical cues in the ancestral

environment taking on a distinct salience. For example, the gestating offspring of gravid field crickets present in an environment with a high density of predatory wolf spiders show an adaptive immobility as adults in the presence of the wolf spider odor [56]. From an olfactory perspective, supplementing the diet of a pregnant mouse female with 'cherry' or 'mint' odors, results in the descendant generations exhibiting a preference for those odors [70]. Complementing this behavioral preference are increased volumes in the olfactory bulbs of the glomeruli that process cherry [M71-expressing olfactory sensory neurons (OSNs) and glomeruli] and mint (M72-expressing cells and glomeruli).

In terms of exposure of ancestral generations to specific environmental cues prior to conception and the effect of such exposure on how descendants might process those cues, less empirical data exist but are beginning to accumulate. First, there is the study that examined the inheritance of behavior toward cocaine in rats after the ancestral generation had been exposed to and self-administered cocaine for 60 days [71]. Counter-intuitively, the F1 male offspring of these F0 animals showed a delayed acquisition to self-administer cocaine themselves. To ascribe a mechanism for this effect, the researchers honed in on the neurotrophin BDNF of which protein and mRNA was found to be increased in the medial prefrontal cortex (mPFC) of the F1 generation. Administration of an agent that blocks BDNF action to the F1 males reversed the effect previously stated. Querying the acetylation status of histones around *BDNF* in the sperm of F1 males revealed specific promoters of *BDNF* being associated with acetyl-H3. This potentially sets up that locus for enhanced transcription, potentially resulting in the increased BDNF levels noted in the mPFC of these animals. Whether these changes could be inherited via the F0 sperm did not seem to be explicitly tested but presents an avenue by which the resistance to self-administer cocaine by the F1 generation after F0 self-administration could be inherited.

Most recently, we used olfactory fear conditioning in mice to ask how a specific environmental cue is perceived and processed by the descendant generations after the ancestral population had been conditioned with that cue [72]. Olfactory fear conditioning results in animals developing a fear toward the odor that was paired with the footshocks. When conditioning occurs using acetophenone, an odor that activates the M71-expressing OSN population in the nose, more M71 neurons are found in the nose of these trained animals and more axons converge into a larger glomerulus in the olfactory bulb [86]. We extended these above findings by mating F0 conditioned animals and then assaying behavior and neuroanatomy in descendant generations that had no prior exposure to the F0 conditioned odors except at the time of behavioral testing. F1 males sired by F0 males conditioned to acetophenone showed an enhanced behavioral sensitivity to acetophenone. The specificity of this F1 response to the F0 conditioning odor was demonstrated by F1 males sired by F0 males conditioned to another odor, propanol, not showing a behavioral sensitivity to acetophenone but only to propanol (Figure 4).

Accompanying this behavioral sensitivity to acetophenone, we found an increased number of M71-expressing OSNs in the nose of the F1 animals and resultant larger glomeruli in the olfactory bulbs. To establish whether either social transmission or biological inheritance was the cause of these effects, we examined the F2 generation, performed IVF with F0 sperm, and conducted cross-fostering studies. All these approaches led us to conclude that our effects were explained by information being inherited via sperm. To investigate the specific nature of this inheritance, we queried the epigenetic landscape around *M71* in the sperm of F0 and F1 males. Chromatin immunoprecipitation (ChIP) on sperm DNA [51] indicated that histone modifications around the *M71* locus in the sperm were not altered as a consequence of conditioning. Bisulfite sequencing of *M71* revealed that the M71 locus is hypo-methylated in F0 sperm as well as F1 sperm when the F0 generation had been conditioned with acetophenone. This could potentially set up the *M71* locus to be transcribed in more quantity in the descendant F1 and F2 generations.

From rodent studies it is currently not possible to define environmental signals and corresponding mechanisms that might be inherited through the gametes to subsequent generations. Both presumably system-wide signals such as the activation of the central stress hormone axis by early life stress [69] as well as very specific activation of the olfactory system [72] have been shown to induce heritable signals. Thus, the qualitative and quantitative properties of environmental signals that can be transmitted through the germline remain elusive at the moment but the studies mentioned above may suggest that different mechanisms may exist with potentially direct effects (e.g., GR activation in sperm) and indirect effects (e.g., activation of the olfactory system with subsequent signal transduction to the germline).

*Inheritance of ancestral environmental exposure in humans.* Transgenerational inheritance of an ancestral exposure to environmental conditions remains a very controversial field in human psychiatric research due to the fact that controlled studies are neither feasible nor ethical and phenotypic as well as biological data across several generations are lacking. Nonetheless, examples of non-behavioral inheritance have been shown to occur in humans as exemplified by exposure to chemical substances, which most likely include the transmission via epigenetic signaling cascades [73]. By contrast, the inheritance of behavioral traits with regard to psychiatric phenotypes and their importance for the development of mental disorders remains contestable.

One of the most prominent examples in this context is the exposure to severe trauma and ancestral PTSD. Non-exposed offspring of trauma exposed individuals are more likely to develop anxiety-related disorders and depression compared to controls [74–76]. Maternal depression and anxiety also influence the risk for psychopathology in offspring [77,78]. Although the mode of transmission is difficult to dissect from the genetic inheritance of risk, evidence suggests a non-genetic inheritance by behavioral transmission or non-genetic, non-behavioral pathways such as epigenetic inheritance. The differentiation between the latter is often impossible to achieve in humans. Given the fact, that most studies are investigating

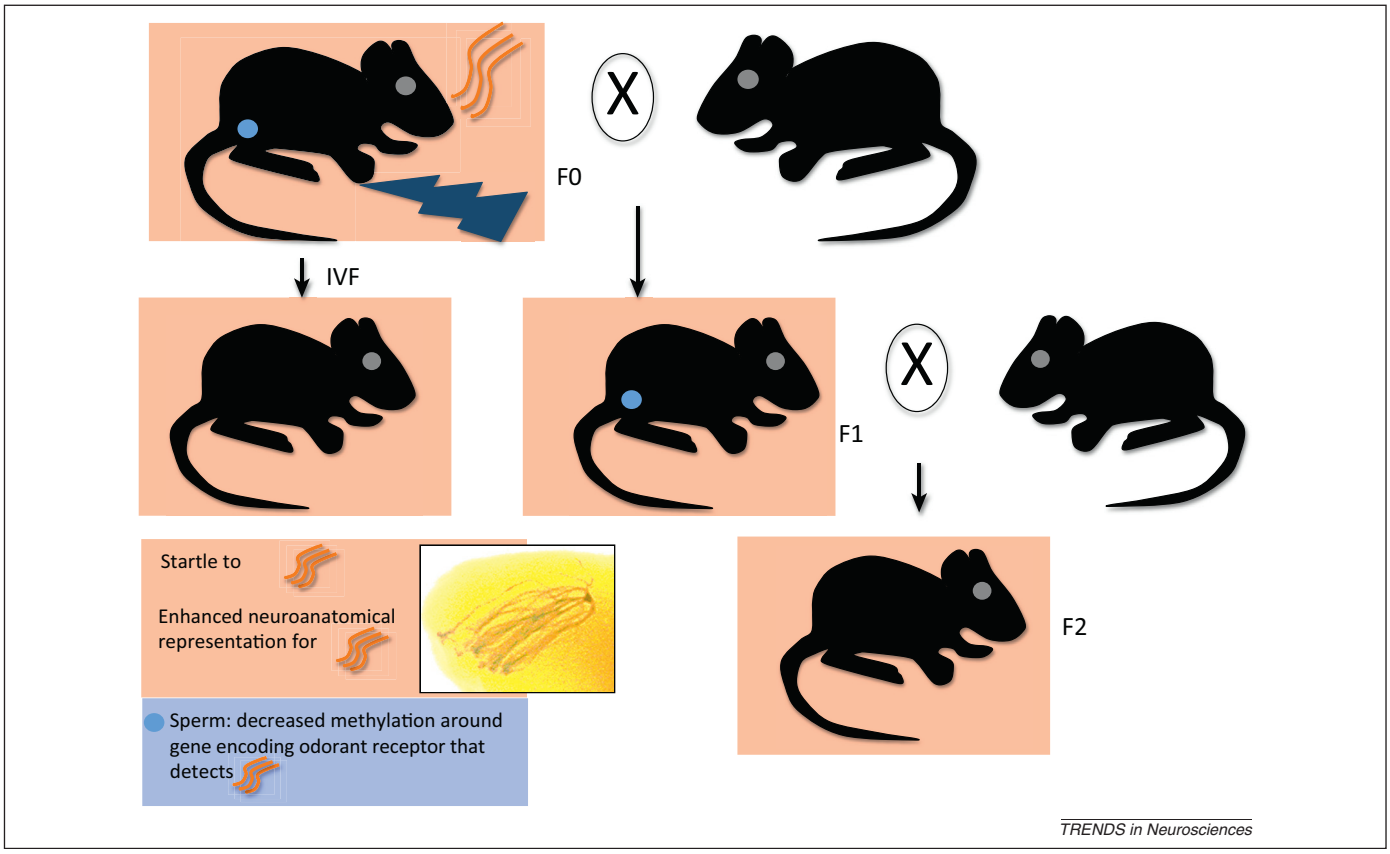


**Figure 4**. Epigenetic transmission of learned olfactory behavior. Training an F0 generation of mice in an olfactory fear conditioning paradigm wherein a particular odor (orange lines) is paired with a mild foot-shock (blue jagged shape) results in a sensitivity toward that odor in the F0 generation that also extends into the F1 and F2 generations that have never been exposed to that odor before. When acetophenone (sensed by M71 receptors) is used as the conditioning odor in the F0 generation, there are more M71-expressing olfactory sensory neurons (OSNs) in the nose of these animals that then results in more axons converging into larger glomeruli in the olfactory bulbs of the brain. This enhanced neuroanatomical representation for M71 is also observed in the F1 and F2 generations, and of note, in a generation created via *in vitro* fertilization (IVF). The persistence of sensitivity to the F0-conditioned odors in F1, F2, and cross-fostered (not depicted) generations implies a biological inheritance of information, as is the case made by the observation of more M71 representation in F1, F2, and IVF-derived generations. We observe decreased DNA methylation around the M71 receptor gene in sperm of the F0 and F1 generations, potentially associated with the enhanced representation for M71 neurons in the descendant generations.

offspring raised by their biological parents, a behavioral transmission of risk by the parent cannot be excluded. Besides a behavioral transmission, it is pivotal to keep in mind that several generations are required to support a potential epigenetic transmission between and across generations [79].

To date, the first studies investigating the epigenetic signatures of ancestral environmental exposure in the field of psychiatry are emerging. An investigation of the $1_F$ promoter of the GR gene with regard to maternal violence exposure found that although the promoter methylation in mothers remain unaffected, offspring GR methylation changed according to the ancestral violence exposure [80]. Further, experiencing the Holocaust has been found to evoke maternal and paternal PTSD influences on the DNA methylation of the $1_F$ GR promoter in unexposed offspring suggesting an intergenerational transmission [81]. Moreover, exposure of pregnant women to the Rwandan genocide resulted in lower cortisol and GR levels with higher $1_F$ GR methylation compared with controls [82].

Other classical examples in humans are periods of severe food depletion as investigated in the Överkalix studies and the Dutch famine winter study [83,84] with regard to metabolic, cardiovascular, and mortality endpoints. These studies highlight the importance of direct exposure to nutrient restriction *in utero* but also transgenerational effects in non-exposed generations.

## Concluding remarks

Within the past decade, the field of epigenetic regulation of gene function has gained great momentum. Remarkably, on the cusp of an era in which genetics was beginning to finally feel 'finite' through the successful sequencing of large mammalian genomes, an entire new era of gene regulation has stepped in, potentially making the workings of genome regulation exponentially more complex than was previously appreciated.

Additionally, our understanding of the neural basis of memory formation has paralleled this epigenetic revolution, and seemingly every mechanism of previously 'understood' synaptic plasticity and neural genetic organization also has a new chapter related to epigenetics to be explored. Even more surprisingly, the newfound understanding of epigenetic modulation has re-opened the possibility of transgenerational inheritance of acquired traits through the process of epigenetic marking in gametes – an exciting but at times overwhelming and certainly controversial idea

(Box 2). Previous examples already exist to demonstrate that behavioral traits may be inherited from ancestral populations. However, nuanced behavioral paradigms and sophisticated reductionistic techniques are paving the way to definitively address the matter of transgenerational inheritance of behavioral traits [85] (Box 3).

This brief review has outlined some of the more well-understood mechanisms of epigenetic regulation, and has provided some examples as to how such regulation may be used by neurons to contribute to the encoding of memory formation. Further, we have provided some very recent examples of how acquired or even learned behaviors may potentially be transmitted via the gametes to alter behavioral responses to the environment in subsequent generations.

The fields of epigenetics and memory formation are in many ways natural complements of each other; however, they have historically been seen as altogether different events. How exciting and ironic if many of the mechanisms that underlie memory within an organism, and perhaps across generations, are due to these same epigenetic processes.

**Box 3. Outstanding questions**

As evidence for the transgenerational inheritance of traits accumulates, this field of research will open up exciting questions that will form the bedrock of how we view behavioral neuroscience. These questions pertain to:

- How is information about environmental stimuli that an organism is exposed to relayed to the gametes? This line of investigation will probably focus on how circulating factors like exosomes and the cargo that they carry impact the gametes. In addition, any direct effect of the environmental stimuli on gametes warrants attention.
- What mechanisms allow for registering of salient environmental experience at specific genetic loci? Small RNA-dependent mechanisms have been shown to underlie several instances of transgenerational inheritance and future work will undoubtedly dissect these details further.
- How do genetic loci marked by environmental stimuli escape the phenomenon of epigenetic reprogramming? If epigenetic marking of genetic loci underlies some aspects of transgenerational inheritance, understanding how these marks escape the process of epigenetic reprogramming soon after fertilization and germ cell development will be crucial.
- Are the mechanisms by which transgenerational inheritance occurs dynamic or static? It is crucial to understand the temporal characteristics of any mechanisms by which genetic loci register information about the environment; for example, over how many generations do the effects persist, do they become fixed in the genome, can they be reversed? Such a strand of questioning will also illuminate the temporal window of vulnerability (e.g., during development, adulthood) during which the genome of an exposed generation is marked by environmental stimuli setting up a potential scenario for epigenetic inheritance.
- How penetrant are the effects of ancestral experiences on descendant genomes and phenotypes? In cases of inheritance via both the maternal and paternal lineages, it will be interesting to compare and contrast what proportion of the descendants are affected as well as the nuances underlying both the differences between affected and unaffected populations and individuals.
- Can information about all environmental stimuli be transmitted or inherited? Both the positive and negative valence of environmental stimuli profoundly affect the exposed population, but are both valences transmitted to or inherited by the descendant generations? Or is there a selectivity?

**Acknowledgments**

Funding for work in the Ressler lab was provided by the National Institutes of Mental Health (MH096764 to K.J.R.), the Howard Hughes Medical Institute, and the Burroughs Wellcome Fund (to K.J.R.). Support was also received from a Cottrell Postdoctoral Fellowship awarded to S.A.M. from the Department of Psychiatry and Behavioral Sciences, Emory School of Medicine. T.K. is supported by an EMBO long-term fellowship.

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