



Review

Nature, nurture and epigenetics

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ABSTRACT

Real life by definition combines heritability (e.g., the legacy of exposures) and experience (e.g. stress during sensitive or 'critical' periods), but how to study or even model this interaction has proven difficult. The hoary concept of evaluating traits according to nature versus nurture continues to persist despite repeated demonstrations that it retards, rather than advances, our understanding of biological processes. Behavioral genetics has proven the obvious, that genes influence behavior and, vice versa, that behavior influences genes. The concept of Genes X Environment (G X E) and its modern variants was viewed as an improvement on nature-nurture but has proven that, except in rare instances, it is not possible to fractionate phenotypes into these constituent elements. The entanglement inherent in terms such as nature-nurture or G X E is a Gordian knot that cannot be dissected or even split. Given that the world today is not what it was less than a century ago, yet the arbitrator (differential survival and reproduction) has stayed constant, *de novo* principles and practices are needed to better predict what the future holds. Put simply, the transformation that is now occurring within and between individuals as a product of global endocrine disruption is quite independent of what has been regarded as evolution by selection. This new perspective should focus on how epigenetic modifications might revise approaches to understand how the phenotype and, in particular its components, is shaped. In this review we summarize the literature in this developing area, focusing on our research on the fungicide vinclozolin.

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1. Introduction

Genetic constitution predisposes, while events experienced throughout in development shape, how males and females respond

Abbreviations: CRS, chronic restraint stress; EDC, endocrine disrupting chemical.

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in adulthood. Individual variation is the substance of evolutionary change, and understanding the organization of variation among individuals is both the original, and the future, frontier in environmental epigenetics. Exactly how different avenues of proximal and ultimate causation combine has been the topic of much interest. The guiding principles for the last 1.5 centuries have been that of selection, heritability, and individual variation proposed by Darwin but developed by others and codified in the Modern Synthesis. These principles successfully accounted for the majority of observations of change within and between species at the time. However, certain foundational aspects of the Modern Synthesis,

particularly the Biological Species Concept of Mayr (1942), has succumbed to further discoveries of the breadth and depth of species diversity (Bush, 2000). Environmental epigenetics appears to be still another exception that defies these principles.

Development is a cumulative process characterized by the emergence of form and function. It is regulated by the subtle concatenation of signaling molecules that act with great precision both in time and space. Comparison of the developmental programs of diverse organisms, the medium of evolutionary developmental biology (Evo-Devo), has made fundamental contributions to understanding the origin and evolution of embryonic development. Particularly relevant to the present review is the phenomenon of phenotypic plasticity (ability for phenotypic change in response to environmental change) and how the environment influences development and evolutionary change.

To the working scientist, the first decision is the selection of when during the life history the measures will be taken. Some studies are longitudinal, but most are much more limited. This becomes important because life is punctuated by periods in which the individual is particularly sensitive to changes in either the internal or external environment. Challenges to the embryo, the newborn, or adolescent can redirect developmental processes with both immediate and life-long consequences. The primary source of these challenges is usually from the environment and, as they interact with the internal milieu, transforms morphological, physiological, and neural traits as well as epigenetic modifications of normal patterns of gene expression. Here the distinction between *molecular* and *molar* epigenetics is important. As defined previously (Crews, 2008): molecular epigenetics refers to gene expression at the transcriptional and translational levels at any given point in time, while molar epigenetics refers to how the individual interacts with its biotic and physical environments through time. Thus, as molecular alterations emerge they result in changes in behavior and neuroendocrine processes, modifying how individuals respond to conspecifics and their environment. This in turn brings about changes at molar levels of biological organization.

The functional unit of evolutionary change is the reproductive success of individuals. The *sine qua non* of reproductive success is the production of offspring that themselves reproduce. While this seems straight forward, it is not. Reproduction is predicated on a number of elements, many of which converge to the final common process of mating. While sexual reproduction and gonochorism (separate sexes in separate organisms) are the most common form of reproduction in animals, they are by no means the only mode and pattern of reproduction. However, for the purposes of this review we focus on gonochoristic vertebrates.

Mating is almost always the consequence of mutual consent by the participating partners. Making the correct choice of a mate has a pronounced impact on reproductive success of both partners. Except in unusual systems, in nature the mating partners choose one another (Carson, 1987, 2003; Crews, 1992; Drickamer et al., 2003; Gowaty, 2007). Experiments with flies, birds, and rodents indicate that individuals who are allowed to select, and be selected by, their mate exhibit greater reproductive success than force-paired animals. This consent is based not only on the internal milieu that motivates each individual to seek a partner, but also on the satisfactory nature of the phenotypic traits the potential mate displays. Therefore, the coordination of egg and sperm maturation and release, complementarity in the signal and receiver, and reciprocity of mounting and lordosis (in rats) are essential preambles for successful reproduction. These synchronizing processes are evident at all levels of biological organization (Crews, 1992) and we extend it here to the level of the epigenome.

Finally, the fact that the sexes develop and respond differently throughout the course of their respective life histories emphasizes that any study on epigenetic modifications needs to incorporate

both males and females. Why is it necessary to emphasize the obvious? It is still common to see in the literature studies on a single sex (extending to the sex genotype of cells). This is a major flaw in both conceptualization and design. Without comparing males and females at the same life stage leaves the discipline with an incomplete understanding at the level of species. This deficiency renders conclusions that apply only to the sex studied and with dubious relevance to the sex not studied. The negative consequences of this practice are readily seen in human health care, prompting the Women's Health Initiative (Anderson et al., 1998). For the authors, understanding sex differences in susceptibility to environmental challenges is particularly important. Sex biases exist in a variety of neurobehavioral disorders in humans, often with significant differences in relative risk level and severity. For example, women have higher levels of diseases and disorders such as Alzheimer's disease, dementia, major depressive disorder, posttraumatic stress disorders, anxiety and panic disorders (Solomon). Disorders such as autism-spectrum disorder and attention deficit hyperactivity disorder predominate in men (Baron-Cohen et al., 2005). Developmental sex differences in adrenal and reproductive hormones must play a role in the etiology of many disorders that are sexually dimorphic in frequency because, in many instances, they manifest following adrenarche/puberty (Christian and Gillies, 1999; Gillies and McArthur, 2010; Goel and Bale, 2009; Shors, 2006; Weinstock, 2007; Wood et al., 2004).

2. Endocrine disrupting chemicals

An unfortunate consequence of the chemical revolution (circa 1940) has been the accumulation in the environment of synthetic chemicals that mimic the action of naturally occurring signaling molecules; these are termed endocrine disrupting chemicals (EDCs) (Diamanti-Kandarakis et al., 2009). Some of the compounds were developed to withstand natural degradation (e.g., polychlorinated biphenyls) while others engender more harmful metabolites (e.g., DDT). Two consequences have been the magnification of concentrations through trophic levels and their global spread, leaving all living organisms today with a body burden of some mixture of EDCs.

The legacy of certain chemical exposures, particularly EDCs, has permanently altered the present and future health of humans and wildlife (Crews and McLachlan, 2006; Diamanti-Kandarakis et al., 2009; Landrigan, 1990; Landrigan and Miodovnik, 2011). These effects are manifest by two different modes (Crews, 2008). The first is via direct exposure or 'context-dependent' modifications. A large number of examples exist of the consequence of exposure to EDCs affecting the life history of individuals and their offspring in both animals and humans. Alternatively, epigenetic modification can be 'germline-dependent' modifications, manifesting each generation in the *absence* of the causative agent. Because the change in the epigenome is permanently incorporated into the germline, such environmental factors have the potential to re-direct the course of evolution.

This paper will review a series of studies directed at illustrating how exposures may influence life history as well as their potential evolutionary impact. First, we will consider how transgenerational germline-dependent modifications interact with context-dependent epigenetic modifications at the level of physiology, behavior, and the brain in adulthood. Second, we will consider how transgenerational modifications might influence traits that have evolutionary consequences.

3. Nomenclature

How interactions are studied in the emerging field of environmental epigenetics is worth mentioning. The data to be discussed can be classified into three categories of effects and are illustrated

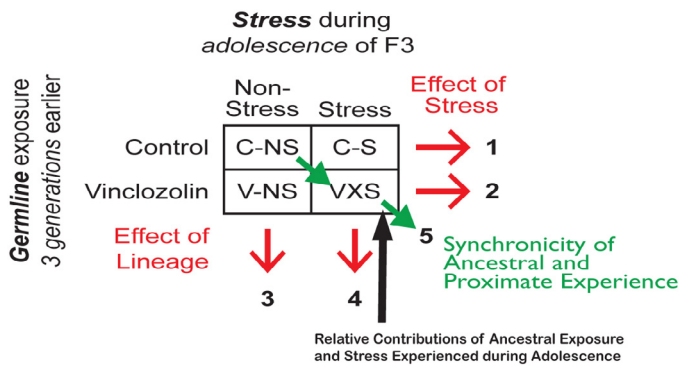


Fig. 1. The “two-hit 3 generations apart” model for examining the effects of germline-dependent and context-dependent epigenetic modifications. Inherited (germline-dependent epigenetic modifications) and experienced (context-dependent epigenetic modifications) challenges act singly and in combination to create new phenotypes. Illustrated are the experimental design and the important comparisons. These are referred to as first (comparison 1 & 3), second (comparison 2 & 4), and third order (comparison 5) effects. Main effects (first order) would be the result of ancestral exposure to vinclozolin or the result of chronic restraint stress (CRS) during adolescence. Interaction effects (second order) refer to those observed when comparing one variable in the context of the other as well as the relative contributions of ancestral exposure to vinclozolin and CRS experienced during adolescence (V X S); the latter is commonly the statistical interaction designated by analysis of variance. The third order effect is the combined effects of important heritable and experienced phenomena that are not causally connected, but may co-occur, resulting in an altered phenotype that cannot be attributed to either the heritable component or the experienced component. Animals experiencing the combined effects of these two events separated by 3 generations, when compared to the control, stress condition animals, reveal the altered phenotype that cannot be attributed to either of the two-hits alone and is not evident in the V X S interaction.

in Fig. 1. First order effects would include the consequences of ancestral exposure to vinclozolin and CRS during adolescence in the descendant animals 3 generations removed and are statistical main effects. Second order effects are the interactions observed when comparing one variable in the context of the other as well as the statistical interaction. In this instance it would be the effect of vinclozolin in animals that have received CRS and the effect of CRS in animals from the vinclozolin-lineage. Traditionally these effects can be additive or synergistic in nature (Crews and Gore, 2011; Berthoud, 2013). Third order effects are of two types: the traditional statistical interaction term yielded by analysis of variance (V X S) that indicates the relative contributions of the two main effects. An alternative comparison is to compare the control animals (in this instance between control, NonStress animals with animals from a vinclozolin-lineage that received CRS during adolescence). This difference between manipulated versus control underscores the exact nature of the changes generated.

There are different types of ‘two-hit’ designs. Classically this takes the form of a single exposure followed by a second exposure after some period of time; e.g., estrogen priming prior to the administration of progesterone to facilitate the expression of sexual behavior in female rats or estrogen priming followed by a second injection of estrogen to stimulate uterine hypertrophy. In a biological sense, the prenatal secretion of gonadal steroids prenatally followed by a second increase in gonadal steroids during puberty is a two-hit process. Most of these types of studies occur within the life history of an individual and the emergent consequences are the focus. This third order effect would be *emergent* in nature as defined by Mayr (1988): “When two entities are combined at a higher level of integration, not all the properties of the new entity are necessarily a logical or predictable consequence of the properties of the components”. Thus, in most instances this term refers to significant sequential events within the life history of an individual (e.g., conception, birth, adolescence, sexual maturity) and, as such, are causally

related because they are stages within a linear path (conception to death).

However, what is not captured in ‘emergent’ is the combined effects of important heritable and experienced phenomena that are not causally connected, particularly when generations separate the hits and the hits are fundamentally different in nature. For example, in transgenerational epigenetic modifications the first hit may be experienced as an embryo in the life history of an individual while the second hit occurs during the life history of descendant generations. An example of this is the ‘two-hit, 3 generations apart’ model we have used. In this instance the hits are different in nature (vinclozolin exposure, a germline-dependent epigenetic modification versus exposure during adolescence to restraint stress, a context-dependent epigenetic modification) and occur in different generations. In this instance there is no causal connection between the exposures (e.g., an ancestral hit may be exposure to an EDC, while the hit to the descendant may be a stressful experience). It is important to understand that the nature of the hits must be different; that is, exposures to different EDCs that operate by different mechanisms of action would share causality in that they operate as EDCs.

We have documented that when acausal factors co-occur in different generations, it will result in an altered phenotype that cannot be attributed to either the heritable component or the experienced component (Gillette et al., 2014). The most appropriate term for this type of third order effect is *synchronicity* or the “the simultaneous occurrence of two meaningful but not causally connected events” (Jung, 1955). Thus, synchronicity reflects the impact of epigenetically induced transgenerational history on how descendants respond to events in their own life, particularly how they perceive challenges. This constitutes a new and different “order” of historical causation.

4. General materials and methods

Although the studies cited have all been published (Crews et al., 2007, 2012; Gillette et al., 2014; Skinner et al., 2014), it is useful to briefly describe the basic experimental design. The most important points here are that: 1) in both series of studies our experimental animals were F3 descendants of control or vinclozolin lineages; 2) we performed behavioral characterization of the rats; 3) we used their brains for transcriptome, gene expression, or metabolic profiling; and 4) we related behavior and brain endpoints within the individual. Sprague-Dawley (SD) rats were bred at Washington State University. An F0 generation of pregnant mothers was injected with vinclozolin (100 mg/kg/day) or DMSO (control) on embryonic days 8–14. Subsequent generations were bred such that there was no sibling or cousin inbreeding. Thus, pups of the F3 generation had no chemical body burden. On weaning (PND 21) these pups were shipped to the University of Texas at Austin the following day.

Two manipulations were used. In the first (**life history impact**) one-half of the male and female F3 control and vinclozolin lineage animals were exposed to chronic restraint stress (CRS) during adolescence for 6 hours per day for 21 days. Hereafter the animals subjected to CRS will be referred to as Stress while those that did not receive CRS will be referred to as NonStress. Behavioral testing was conducted two months later when animals were adults. In these studies standard behavioral tests were administered to each individual. Tests of sociality and social memory included sociability and social preference. Tests of anxiety included open field, light–dark transitions, and elevated plus maze. After all behavioral testing was completed animals were euthanized to enable us to relate behavioral outcomes to underlying brain changes. This was accomplished by microdissection of brains to enable precise neuroanatomical specificity.

In the second series of studies (**evolutionary impact**) males and females were produced as described above but without any further

manipulation in the F3 generation (i.e., no CRS). In this instance individuals were allowed to grow and develop into adults in dyads (same sex but of opposite lineages). As adults the animals were given a partner preference test consisting of placing an individual (male or female) in the center of a large, three-chamber glass-testing arena. At either end was a compartment containing opposite-sex stimulus rats separated by a wire-mesh barrier to allow exchange olfactory, visual, and tactile cues. Both the experimental rat (chooser) and the two stimulus rats (“choosees”) were F3 vinclozolin or control descendants. When a female was the chooser, one choosee was an F3 vinclozolin male and the other an F3 control male. When a male was the chooser, one choosee was an F3 vinclozolin female and the other an F3 control female. In other words, we tested the ability of an experimental rat to distinguish between F3 control and vinclozolin opposite-sex partners based on behaviors, scents, and vocalizations of the choosees.

Progress in behavioral neuroscience requires clear connections between established and accepted methods within the discipline and alternative and advanced techniques developed from other disciplines. This chain of evidence is essential to integrating state-of-the-art methods into experimental approaches. In this instance we provide a roadmap that connects morphological and physiological changes with behavioral changes and associated changes in metabolic activity and gene expression in a network of brain nuclei to the transcriptome and methylome of those nuclei. To establish this bridge we first examined the concordance of gene expression patterns between results obtained by PCR-based targeted low-density arrays of known genes and genome-wide transcriptome analysis in specific brain regions. Finally, in preliminary studies global DNA methylation levels were determined for each brain region. In all of the experiments linear discrimination analysis, principal component analysis, and functional landscape analysis were performed.

5. Life history impact

These studies were designed to determine how events experienced during an individual’s life might interact with ancestral exposure to an EDC. We chose CRS because it is exceptionally well characterized at the physiological, neuroendocrine, and behavioral levels in rats. Because the animals were shipped immediately after weaning, all individuals received shipment stress. Weaning signals the transition from dependence on the mother to independence. This initiates the period of adolescence that, in the rat, can be considered to last from PND 22–42. We capitalized on this fact because an emerging literature indicates that adolescence (rather than simply puberty) is a sensitive (critical) period for the development of adult sociality and stress reactivity.

We discovered large sex differences in a variety of phenotypic traits among control NonStress animals (Gillette et al., 2014). This is exemplified by the sex and treatment differences in circulating levels of corticosterone (Fig. 2). As per the literature, the baseline levels of corticosterone are different in control, NonStress males and females, with males having significantly lower circulating levels than females (dashed line in Fig. 2). Stress during adolescence decreases corticosterone in both sexes, with males being affected more than females. The effect of ancestral exposure to vinclozolin is modest in both sexes, but the sex difference is maintained. When vinclozolin, Stress animals are compared to control, NonStress animals, however, a striking difference is seen. That is, ancestral exposure to vinclozolin significantly increases the effects of CRS during adolescence in the descendant females, but there is no apparent effect in males. This means that when these two types of epigenetic modifications are combined, there is a profound sex difference in the scope and nature of reactivity that cannot be explained by either variable alone.

Males and females also exhibit significantly different reactivity profiles, with females showing more anxiety-like behavior. The only

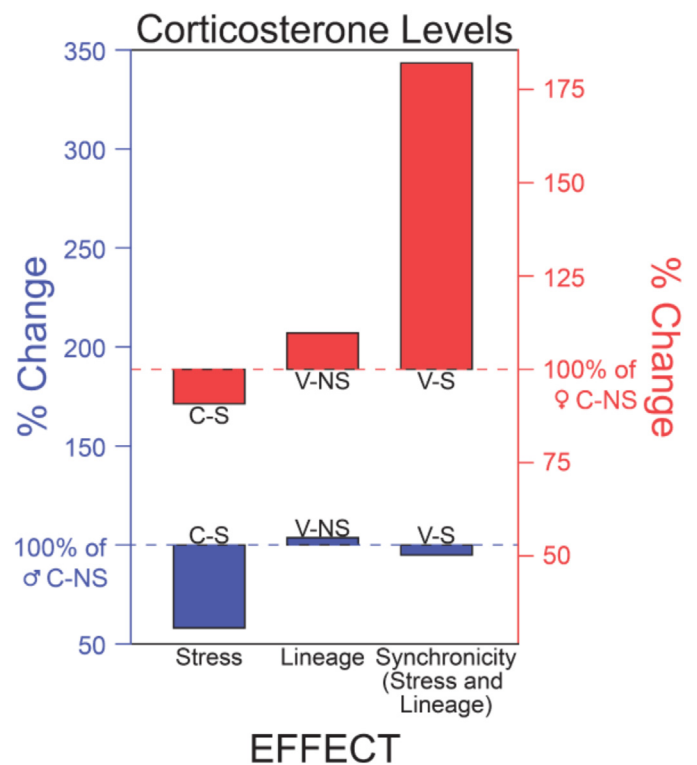


Fig. 2. Exposure to vinclozolin 3 generations previously changes circulating concentration of corticosterone in adult male and female rats. The effect of chronic restraint stress during adolescence (stress) within control-lineage animals is illustrated in the left column. The effect of ancestral exposure to vinclozolin 3 generations previously (lineage) is evident in the middle column. The effect of ancestral exposure to vinclozolin and chronic restraint stress during adolescence compared to control-NonStress animals is in the right column. As expected, control, NonStress females (red) have significantly higher circulating concentrations of CORT relative to males (blue). Comparison of V&S to C-NS animals reveals a striking sex difference is seen; that is, ancestral exposure to vinclozolin significantly increases the effects of CRS during adolescence in the descendant females, but there is no apparent effect in males. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

exception is the behavior of males in the elevated plus maze. Males show a stronger preference for social affiliation than do females, but both sexes prefer to associate with a stimulus animal versus an empty chamber. Only females exhibit a clear preference for the novel stimulus animal when given the choice to investigate a familiar stimulus animal or an unfamiliar stimulus animal.

There is a substantial difference in the profile of cytochrome oxidase abundance in target nuclei, with females showing elevated activity in most nuclei. In general, control NonStress males show decreased metabolic activity in hippocampal nuclei while females exhibit increased metabolism in the medial and central amygdaloid nuclei.

Analysis of the specificity of gene expression according to sex and brain nucleus reveal a marked sex difference in the numbers of genes regulated, with control, NonStress females showing most (16/18) changes in regulation in the CA3 of the hippocampus (CA3), while in control, NonStress males the majority of genes showing changes are in the basolateral amygdala (BLA), bed nucleus of the stria terminalis (BnST), and CA3 of the hippocampus (8/10). The greatest sex difference is found in the pattern of gene expression in the CA3 of the hippocampus of females. Interestingly, the only gene expressed in both males and females (*Mc4r*) was in this group, showing downregulation in males and upregulation in females in the ventromedial nucleus of the hypothalamus (VMH) (Fig. 3).

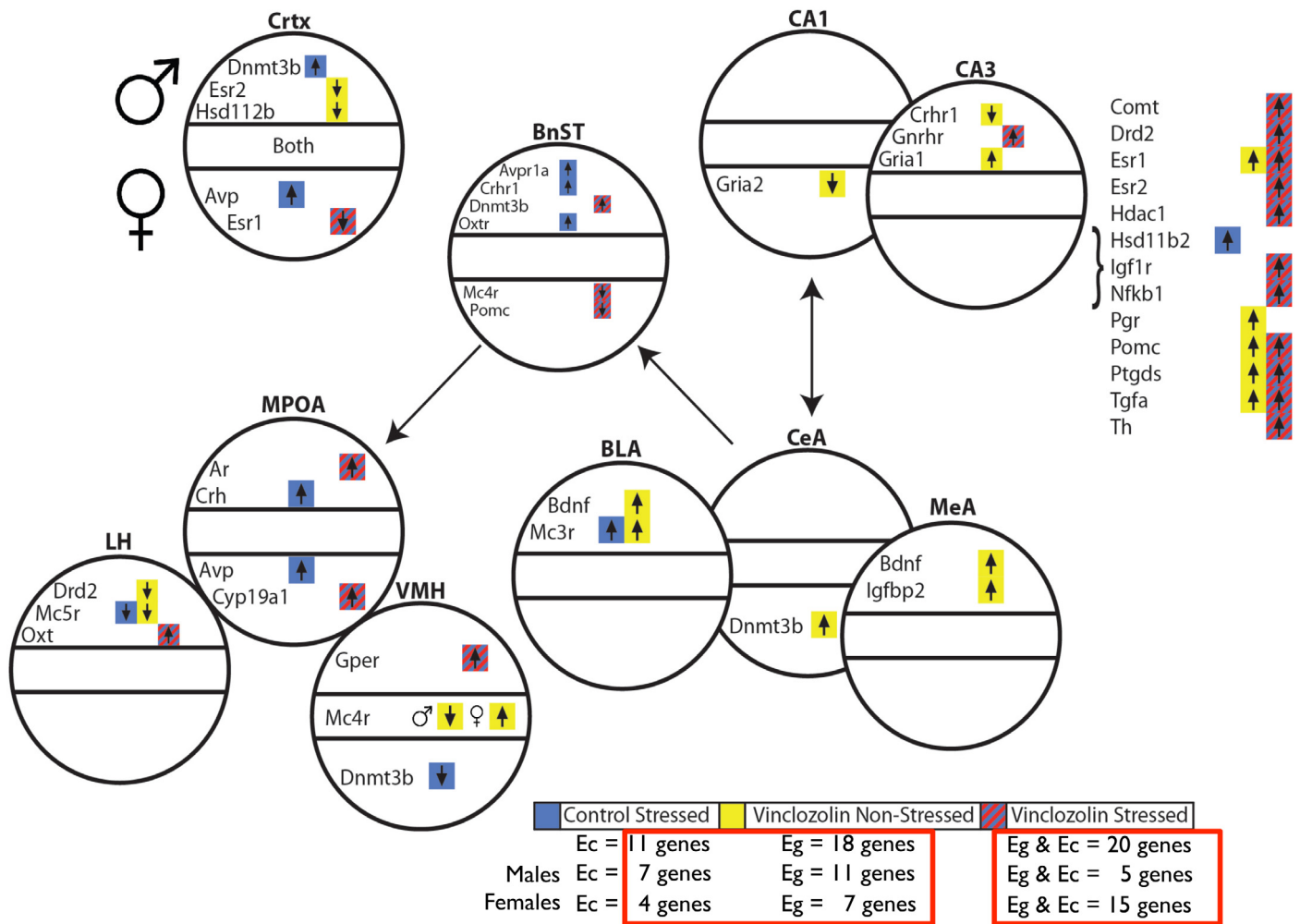


Fig. 3. Sex differences in the pattern of expression in targeted genes in a neural network of brain nuclei involved in social, affiliative, and anxiety-related behavioral tests via Taqman low-density PCR arrays (TLDA). Six limbic nuclei are represented. All differences are equal to or greater than a two-fold difference relative to control, NonStress (C-NS) male and female rats. Note that each region has unique gene expression changes, with a subset of genes identified in multiple nuclei (*Avp* in males; *Esr1* and *Pomc* in females). Region abbreviations: CA3 and CA1 – areas of the hippocampus, CeAmy – central amygdaloid nucleus; BLA – basolateral amygdaloid nucleus; BnST – bed nucleus of the stria terminalis; LH – lateral hypothalamic nuclei. Gene abbreviations: *Ar* – androgen receptor, *Avp* – arginine vasopressin, *Bdnf* – brain-derived neurotrophic factor, *Drd2* – dopamine receptor D2, *Esr1* – estrogen receptor alpha, *Esr2* – estrogen receptor beta, *Gnrhr* – gonadotropin releasing hormone, *Lepr* – leptin receptor, *Mc4r* – melanocortin 4 receptor, *Negr1* – neuronal growth factor, *Oxt* – oxytocin prepropeptide, *Pomc* – proopiomelanocortin, *Pgr* – progesterone receptor, *Ptgds* – prostaglandin D2 synthase, *Tgfa* – transforming growth factor alpha, *Th* – tyrosine hydroxylase. Green shaded numbers indicate up-regulation level of significance (two-tailed). Red shaded numbers indicate down-regulation and level of significance (two-tailed). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The majority of genes affected belong to receptor class proteins and growth factors. Genes coding for ER α (*Esr1*) and ER β (*Esr2*) are upregulated when the ancestral exposure to vinclozolin and CRS during adolescence of the F3 are combined; ancestral exposure to vinclozolin alone affects only *Esr1*. Both *Esr1* and *Esr2* have been implicated in fast neuronal modulation that contribute to learning and memory in female rats (Huang and Woolley, 2012; Smejkalova and Woolley, 2010). That *Esr1* is affected in vinclozolin lineage animals regardless of CRS exposure suggests that its expression may be affected by altered methylation patterns established in germline cells during embryonic vinclozolin exposure of the F0 generation (see below). Why this effect would be limited to the CA3 of the hippocampus is not known but could be due to the particular context in which the gene is expressed. In the vinclozolin lineage, regardless of CRS exposure, arginine vasopressin peptide (*Avp*) is affected in the male lateral hypothalamus; arginine vasopressin is released to the lateral hypothalamus and is suspected to be involved in the control water and food intake and anxiety-related behaviors (Aoyagi et al., 2007; Zelena et al., 2008). The upregulation of the AVP in the

lateral hypothalamus may contribute to the dysregulated weight gain seen in vinclozolin lineage males.

Pomc is post-transcriptionally processed to multiple peptides important in stress signaling, feeding, and sexual behaviors. *Pomc* is downregulated in the lateral hypothalamus both in control and vinclozolin, Stress females. A similar decrease of *Pomc*, along with increased α -MSH production, in young male rats has been reported using another form of chronic stress (5 days housed in cages partially filled with water) (Ogawa et al., 2009). Both *Pomc* and α -MSH are integral to the stress-signaling pathway and can lead to excessive feeding behavior (Maniam and Morris, 2012; Tsujii and Bray, 1989). The present observation of long term effects, extending from adolescence into adulthood, is indicative of a long-term impairment of the hypothalamic–pituitary–adrenal axis. Further, downregulated *Pomc* was seen only in females, suggesting a sex difference in the response to CRS.

It is still rare to find studies that relate the concordance of gene expression patterns between results obtained by PCR-based targeted low-density arrays of known genes and genome-wide

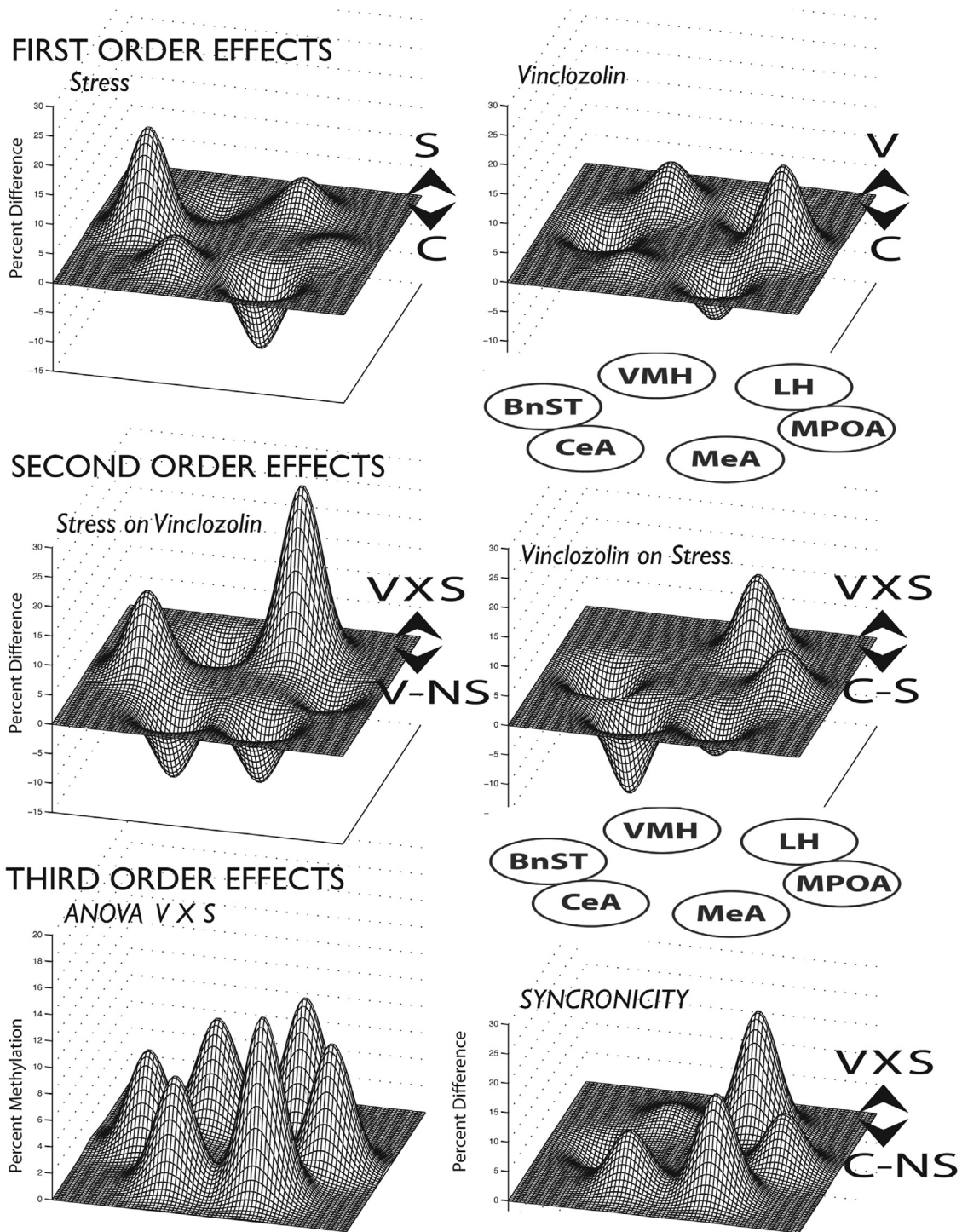


Fig. 4. Changes in global methylation in a network of six brain nuclei are shown using functional landscapes. Each landscape represents the percent change in average global methylation levels for each brain nucleus. Peaks indicate higher levels in the group indicated while valleys represent higher levels in the control group (see Fig. 1). Illustrated are first, second, and third order effects. First order effects (top row) are statistical main effects, in this instance the consequences of CRS during adolescence (top left) or ancestral exposure to vinclozolin (top right) in the descendant animals 3 generations removed. Second order effects (middle row) are the interactions observed when comparing one variable in the context of the other, in this instance the effect of CRS in animals from the vinclozolin-lineage (middle left) or the effect of vinclozolin in animals that had received CRS (middle right). Finally, Third orders effects (bottom row) are of two types: interaction (V X S) from analysis of variance (bottom left) or synchronicity (bottom right). Note, in the first instance represented is the percent in average methylation levels. In other words, the interaction term simply indicates whether the variables contribute significantly to the variance. The latter instance is *synchronicity* representing the difference between vinclozolin animals in relation to control, NonStress individuals. The combined effects of important heritable and experienced phenomena are not causally connected, but co-occur, resulting in an altered phenotype that cannot be attributed to either the heritable component or the experienced component. The nodes are equivalent to brain nuclei (shown as insets) in clockwise fashion: lateral hypothalamus (LH), medial preoptic area (MPOA), medial amygdaloid nucleus (MeA), central amygdaloid nucleus (CeA), bed nucleus of the stria terminalis (BnST), and the ventromedial nucleus of the hypothalamus (VMH).

transcriptome analysis in specific brain regions. Using a rigorous cutoff of two-fold change in expression in both techniques identified potentially important parallels between the two methods of analysis. The same four brain nuclei (basolateral amygdala, bed nucleus of the stria terminalis, central amygdala, and CA3 of the hippocampus) were examined. In general, there was no concordance of genes in the Stress condition. The following genes were both upregulated and concordant: *Bdnf*, *Drd2*, *Igfb2*, *Oxt*, and *Ptgds*. Only one gene (*Igfb2*) was concordant in both males and females. In males all concordant genes were in hypothalamic nuclei with most in the Stress animals (*Oxt* and *Ptgds* in the bed nucleus of the stria terminalis and central amygdala, respectively) or vinclozolin lineage animal (*Igfb2* in the basolateral amygdala) group; the exception was for *Bdnf* in the central amygdala in the vinclozolin, Stress animals. In females most of the concordant genes (*Crhr1*, *Drd2*, *Igf1*, *Igfb2*, *Igf55* and *Ptgds*) were in the CA3 of the hippocampus.

Global 5-methylcytosine measurement of the DNA was performed in order to assess methylation status. The following brain nuclei were measured: lateral hypothalamus (LH), medial amygdaloid nucleus (MeAmy), central amygdaloid nucleus (CeAmy), bed nucleus of the stria terminalis (BnST), and the ventromedial nucleus of the hypothalamus (VMH). A main principal component analysis accounts for 67% of the variation. The primary principal component, which accounts for 27% of the variance, has a correlated increase of BNST and VMH with a decrease in MeA, CeA and MPOA. The second principal component, which accounts for 23%

of the variance, shows that there is a correlation between an increase in BNST, MeA and CeA with a decrease in LH. The third principal component, which accounts for 17% of the variance, shows that there is a decrease in the LH and CeA correlated with an increase in the MPOA. Fig. 4 depicts these various effects in the form of functional landscape analysis (Scarpino et al., 2014) showing the changing relationships in the methylome of brain nuclei change in the first (individual effects), second (interaction effects), and third (synchronicity) order comparisons.

6. Evolutionary impact

While life history effects often have important consequences at the level of the individual, there is little information on how such exposures may affect evolution *per se*. It is the case that allelic (or mutation) frequencies via classic genetic inheritance mechanisms may ultimately change as the organism adapts to persistent causative agents in the environment (Crews, 2008; Kidd et al., 2007; Whitehead et al., 2011), but there is only limited evidence to support this conclusion. The other reason is that in studies using conventional animal models such as commercially bred rats there is no natural or sexual selection operating on reproductive success. In studies with animals in nature the focal animals are those few individuals who survive to become breeding adults. Information on why these individuals survived, while others died (i.e., their life history experiences), is completely lacking.

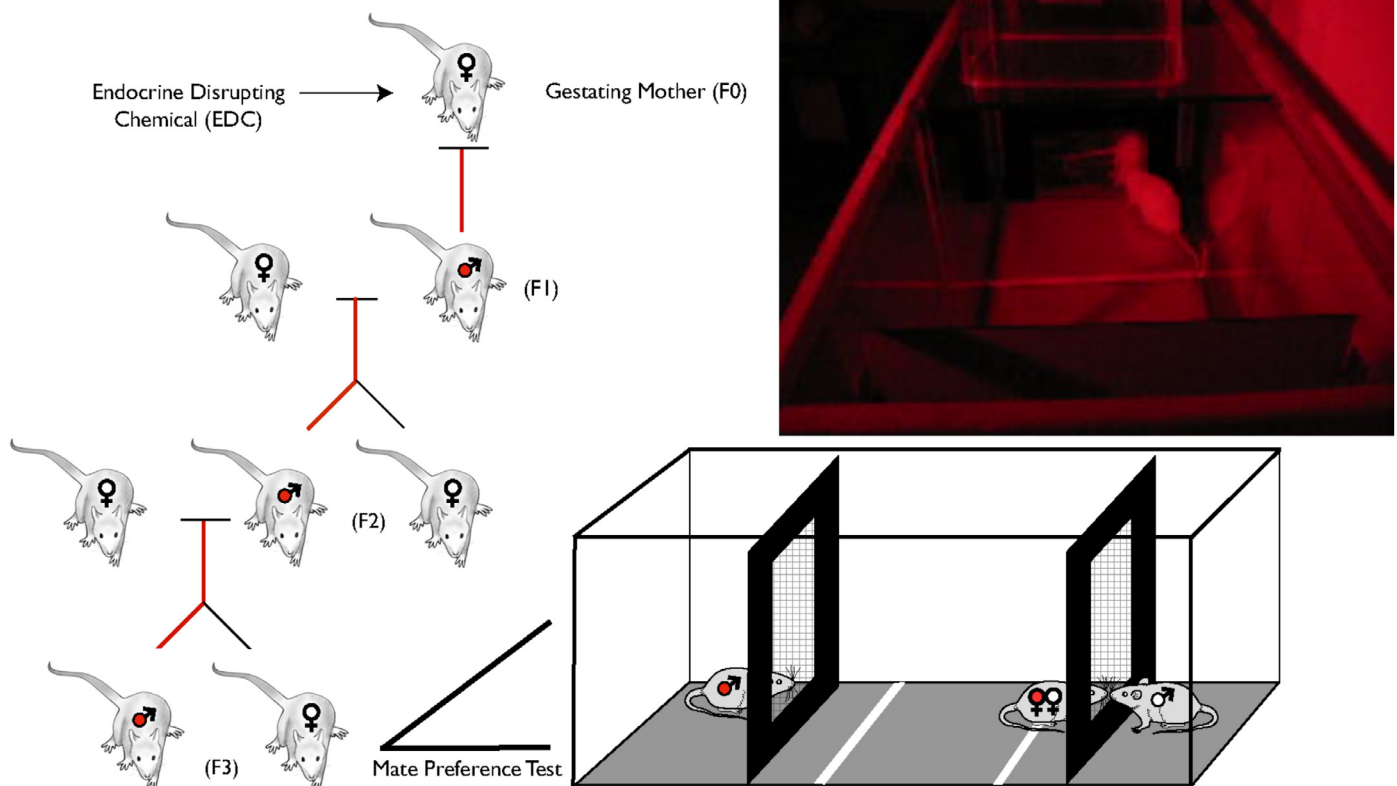


Fig. 5. Determination of a transgenerational epigenetic imprint on mate preference behavior. The left panel shows that 3 generations separate the gestational exposure to vinclozolin. The upper right panel is a picture (under red light when testing occurred) of an experimental animal showing facial investigation of the stimulus animal). The lower right panel is a schematic of the testing apparatus for mate preference. Third generation females from the vinclozolin lineage and the control (DMSO) lineage were tested with males from both lineages in simultaneous mate preference tests; males from the vinclozolin lineage (indicated by red-filled male symbols) and the control lineage (not shown) were similarly tested with females of both stimulus types. The experimental animal (here a female) was placed in the center of the chamber; a stimulus male from each lineage type was at each end of the apparatus. The females could move freely in their chamber but were separated from the stimulus males by a wire mesh. This enabled the animals to communicate by olfactory, pheromonal, or behavioral cues, but physical interaction was limited to touching across the wire mesh. (Modified from Anway and Skinner, 2006 and Crews et al., 2007). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

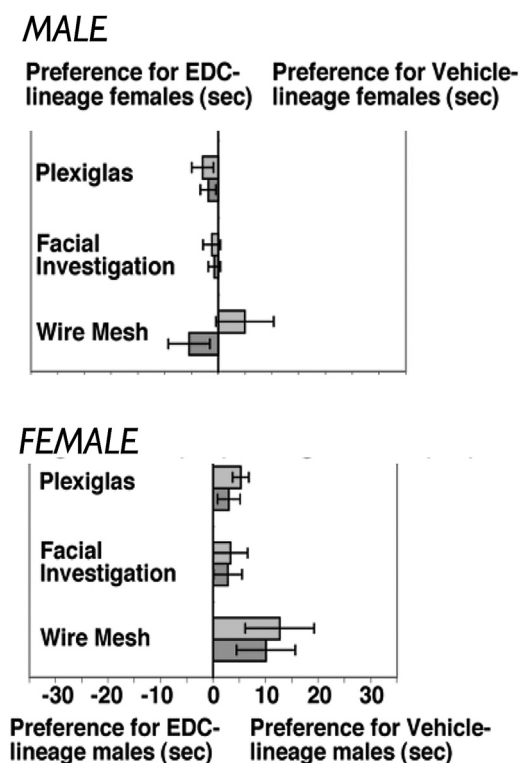


Fig. 6. Third-generation female rats whose progenitors were exposed to vinclozolin are epigenetically altered and prefer males from the unexposed control-lineage. Males do not show this preference. Both females and males from control (DMSO) lineage and vinclozolin lineage were tested with pairs of control- and vinclozolin-lineage stimulus partners. Average differences in the time spent in three behaviors directed to stimulus animal (Plexiglas, facial investigation, and wire mesh). Top panel: behaviors exhibited by males from control- and vinclozolin-lineages toward females from control-lineage (positive, right side) and vinclozolin-lineage (negative, left side). Bottom panel: behaviors exhibited by females from control- and vinclozolin-lineages toward males from control-lineage (positive, right side) and vinclozolin-lineage (negative, left side). (Modified from Crews et al., 2007).

For the purposes of this review, we will focus on the three behaviors involved with investigation of the stimulus animal, namely ‘facial investigation’ which entails actual nose-to-nose contact by the two animals through the wire mesh (see insert in Fig. 5), ‘wire mesh’ in which the experimental animal investigates the stimulus animal directly through the wire mesh, and ‘Plexiglas’, which connotes the experimental animal investigating the area immediately bordering the wire mesh that separated the experimental animal from the stimulus animal.

In the initial study, females from both the vinclozolin and the control lineages discriminated and preferred to associate with male descendants of the control lineage relative to males of the vinclozolin lineage (Fig. 6) (Crews et al., 2007). That is, in the partner preference tests, F3 generation females of both the vinclozolin- and control-lineages discriminate and prefer males who do not have a history of exposure. This is not the case with males that, regardless of lineage, do not exhibit a preference for females from one lineage over the other. Nor is the case that ancestral to vinclozolin affects the ability to discriminate odors; males and females of both lineages explore odors of the opposite sex much more than familiar (self) odors or novel odors of the same sex, and all animals explore novel odors of the same sex more than their own odors.

To explore how these behavioral differences are reflected in the brain, we chose regions known to be associated with mate preference in rodents. Importantly, these regions were dissected rather than punched as in the above studies. The regions included the olfactory bulbs, preoptic area–anterior hypothalamus, amygdala,

hippocampus, and entorhinal and cingulate cortices. RNA was prepared for microarray transcriptome analysis from each brain region independently. The array data were then processed to identify the differentially expressed gene sets for each brain region (=“Signature lists”) (Skinner et al., 2014). The transcriptome alterations were statistically correlated with changes in mate preference behaviors.

Males and females have an approximately equal number of differentially expressed genes in the brain regions. With a single exception (overlap between the cingulate cortex and olfactory bulb in the female), each Signature list is distinct from each other and between the sexes. The most highly represented pathways in the male and female include the olfactory transduction signaling pathway, MAPK pathway, neuroactive ligand–receptor interactions, and axon guidance. Bionetwork cluster analysis of the differentially expressed genes in the various brain regions identify gene modules in each sex. Network analysis reveals separate networks with coordinated and interconnected relationships (i.e. connectivity). The female gene subnetwork indicates angiogenesis, growth, and apoptosis are predominantly affected; in the male apoptosis is the cellular process most affected. Female mate preference behaviors are associated with gene modules in the female amygdala and entorhinal cortex. In rodents females are the discriminating sex and distinguish between males largely on the basis of MHC and olfactory gene products. Our study affirms that the olfactory transduction pathway is a key player. This altered mate preference behavior suggests the existence of an environmentally altered epigenetic transgenerational inheritance of mate preference behavior (Fig. 7).

7. Discussion

Environmental and social stressors are the primary source of epigenetic modifications (Hunter and McEwen, 2013; Karatsoreos and McEwen, 2013; McEwen, 2012). Environmental exposures to EDCs are a contributing factor in the increased incidence of obesity, illness and affective disorders (Crews and McLachlan, 2006; Grandjean and Landrigan, 2006; Landrigan et al., 2012). Chronic or excessive stress during sensitive periods predisposes individuals to develop diseases and disorders later in life (Heindel). The most thoroughly studied sensitive periods in terms of stress are embryonic life and early infancy (Meaney, 2001). Recent studies indicate that stress during adolescence also has enduring effects that include neural remodeling, impaired learning and memory, sensitivity to drugs of abuse, and emotional disorders in adulthood (Jankford et al., 2011; McCormick and Mathews, 2010; McCormick et al., 2010; McEwen, 2010; Romeo et al., 2009; Romeo, 2010; Wei et al., 2011).

Typically, investigations of genetic, neural, behavioral or endocrinological correlates have focused on these units in isolation; a single gene, brain nucleus, behavior, or hormone has been the topic of study following manipulations. Even when these different endpoints are combined, do they tell us much about systems? Genes and their products never act in isolation but operate within a context of a genetic, physiological, and psychological milieu that change in predictable ways as the individual passes through successive life stages. The same can be said of brain nuclei; that is, nuclei function within circuits. Similarly, any consideration of the role of hormones requires understanding the endocrine milieu both past and present.

Previous studies on the transgenerational effects of EDCs such as vinclozolin (Crews et al., 2007) and bisphenol-A (Wolstenholme et al., 2012) indicate that both behavior and their underlying neuroendocrine networks are altered. Our work reveals that such ancestral exposure and present life challenges (CRS during adolescence) interact to compromise adult phenotype (Crews et al., 2012). The elements of these effects have been characterized in both male

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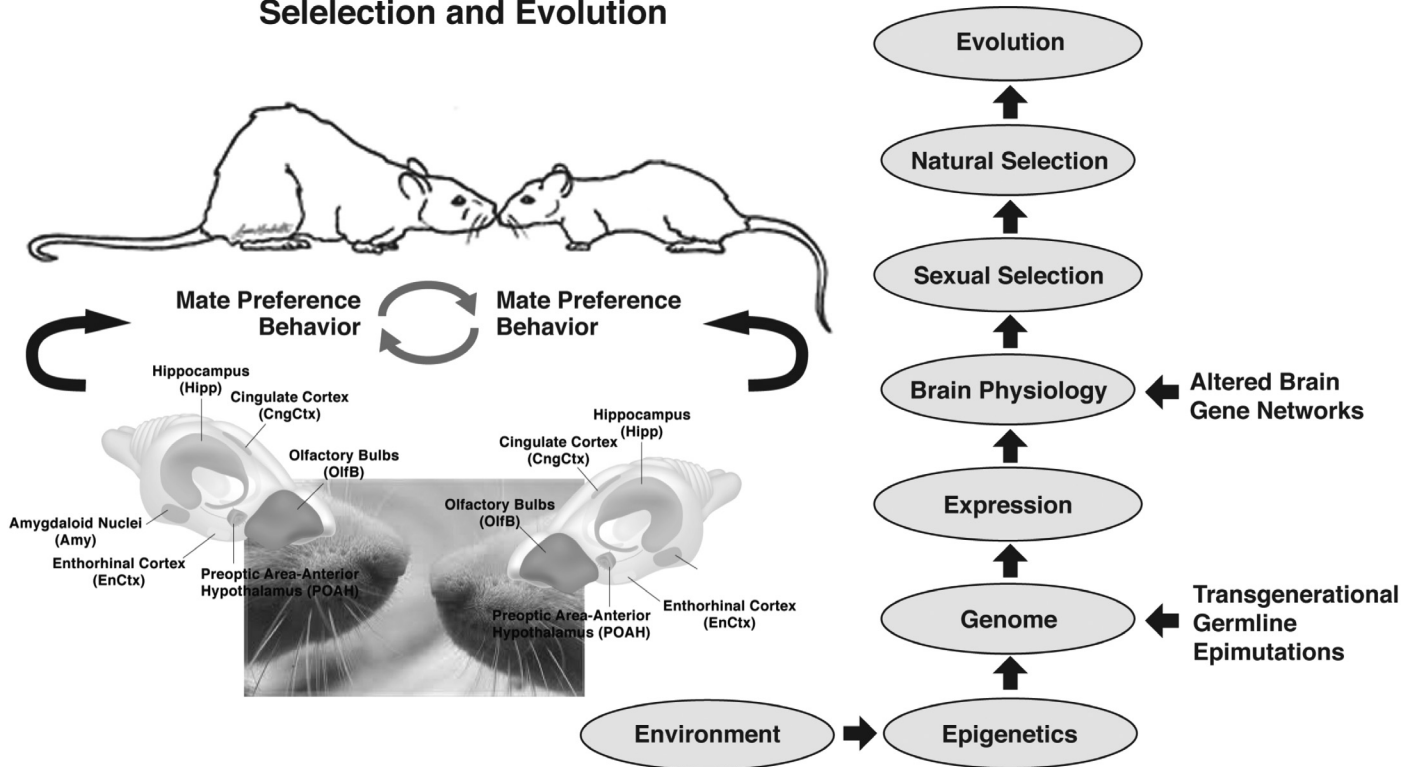


Fig. 7. Studies integrating mate preference behaviors and, in particular facial investigation (upper left panel), brain nuclei and circuits and the patterns of gene expression and transcriptomics therein (lower left panel), lead to better understanding of how the environment influences epigenetic modifications that lead to genomic, physiological and neuroendocrine changes that may influence evolutionary trajectories (right panel). (Modified from Skinner et al., 2014).

and female rats in an expanded battery of anxiety-related and sociality tasks and related these to the underlying metabolic history and patterns of gene expression of the neural network.

This “two-hit 3 generations apart” model enables examination of how germline- and context-dependent epigenetic modifications (vis-à-vis nature versus nurture) might combine and, further, how the sexes might differ in reactivity. This design further illuminates how individually, and together, epigenetic modifications transform physiology, behavior, as well as metabolic activity, gene expression and transcriptome in discrete brain nuclei in a sexually dimorphic manner. We have established that males and female rats differ markedly in stress reactivity when such heritable and proximate life stressors converge. Under these conditions females as adults showed a substantial elevation in circulating corticosterone levels and significantly different reactivity profiles, with females showing more anxiety-like behavior. Females also show elevated metabolic activity in the targeted limbic nuclei and exhibit a significant increase in both number and activity of upregulated genes in the CA3 of the hippocampus. This may have relevance to the marked sex differences observed in human mental disorders (Franklin et al., 2012; Gillies and McArthur, 2010). Women are more likely to manifest stress-related disorders such as anxiety and depression, conditions believed to be predisposed by adrenal glucocorticoid and gonadal hormone secretion (Carvalho-Netto et al., 2011; Solomon and Herman, 2009). For example, ER β plays a pivotal role in the hypothalamic–pituitary–adrenal axis (Handa et al., 1994; Solomon and Herman, 2009; Weiser and Handa, 2009). In our study females had elevated levels of corticosterone and estradiol in the circulation and expression of *Esr2* in the hippocampus. *Esr2* in the hippocampus influences cognition via a fast acting neuronal mechanism (Smejkalova and Woolley, 2010). The higher levels of *Crh* and

Crhr1, and downregulation of *Esr2*, in the central amygdala transcriptome of females are consistent with the literature (Weiser and Handa, 2009; Weiser et al., 2008, 2010). Another gene implicated in human and rodent anxiety disorders is *Ptgds* (Donner et al., 2009; Hovatta et al., 2005; Le-Niculescu et al., 2011; Yamaguchi et al., 2006), which we found exhibits a two-fold increase in the CA3 of the hippocampus of Stress females, and not in males.

In today's world, events are being forced upon biological systems and driving evolutionary change in a manner that cannot be anticipated by classical evolutionary theory. Recent concepts of the exposome (the sum total of environmental exposures in the life cycle (Lioy and Rappaport, 2011) and allostasis (process of achieving stability through change) (McEwen, 2012) do not capture the vital nature of how males and females may respond differently to the same stimuli. The promising reaction scope model (Romero et al., 2009) borrows heavily from the established reaction norm concept fundamental to phenotypic plasticity (Gilbert and Epel, 2009; Pigliucci, 2010; Pigliucci et al., 2006). All of these concepts, however, lack the fundamental ingredient of generational carry-forward, or potentially cumulative, effects.

We have demonstrated that alterations result from experiences within the individual's life history combined with the exposure to EDCs 3 generations removed. The convergence of two life stressors separated by 3 generations (a single exposure to the fungicide vinclozolin prenatally in the ancestral female and CRS experienced during adolescence of the F3 descendants) reveals that females are significantly more vulnerable than males. Debilitating effects occur at all levels of the phenotype, including physiology, behavior, as well as metabolism, gene expression, and genome-wide transcriptome modifications in specific brain nuclei. This, in turn, modifies how descendants of these progenitor individuals per-

ceive and respond to stress. It is noteworthy that females are affected more than males in terms of anxiety but not sociality. Indeed, males tend to be more affected by exposure *or* stress, while females are affected by exposure *and* stress. Thus, the pertinent comparison is not the relative contributions of heredity and experience (or nature and nurture), but how individuals are changed by these events. As such, demonstration of synchronicity does not identify a 'mechanism', a seeming gold standard for some investigators. Rather, we identify a process (synchronicity) whose consequences of two very different insults separated by generations combine to change phenotypic traits at all levels of biological organization. We suggest further this to be the root of the exponential increase in morbidity observed in the last half century.

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