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Epigenetics and Animal Behavior

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INTRODUCTION

The tendency to succumb to the seduction of dichotomies in lieu of grappling with the reality of complexity is human. This is best seen in the nature/nurture debate that dates at least to the ancient Greeks and is reprised every generation in science under various guises (e.g., innate versus acquired, instinct versus learned). And with every generation there is a refutation of this false dichotomy as being sterile, yielding no useful offspring in our knowledge base (e.g., Lehrman, 1970; Gottleib, 2002; Bateson & Gluckman, 2011). This seemingly endless rediscovery is perhaps a good thing as it forces scientists to develop new ways of investigating and illustrating that nothing in biology and psychology is simple. In many ways, the rediscovery of ***epigenetics*** refreshes the debate and, perhaps for the first time, offers a solution.

Put simply, *epigenetics* refers to traits that are not determined by traditional molecular bases for inheritance. A more precise definition would be that ***epigenetic effects*** are changes in the phenotype and/or specific traits that result from the environmental modification of the molecular factors and processes around DNA that regulate genome activity yet are independent of the DNA sequence. Note that the term *environment* is inclusive of all stimuli that may impinge on the organism during its life cycle. How researchers have interpreted epigenetics reflects its dual origins and the nature of the question being posed. At a basic level it is useful to differentiate ***molecular epigenetics*** and ***molar epigenetics***, or bottom-up versus top-down epigenetics. The former perspective

has emerged within the last 25 years from modern genetics and molecular biology and focuses on molecular levels of analysis. The latter perspective has a deeper history, preceding the rediscovery of Mendel's studies, and focuses on questions of evolution and adaptive significance as evident in psychobiology and evolutionary biology. Thus, the object of study in molecular epigenetics is transcriptional and translational control during embryonic development, while in molar epigenetics it is the individual's interactions with its biotic and physical environment through time. More will be said about this distinction later.

Another distinction that must be made is that between ***environmentally induced epigenetic modifications*** and ***parental genomic imprinting***, forms of epigenetic marks that are carried in the male and female germlines. Parental genomic imprints refer to genes that are expressed in a parent-of-origin fashion, that is, from the mother (***maternal imprint***) or from the father (***paternal imprint***). Both environmentally induced epigenetic modifications and parental genomic imprinting involve methylation and histone modifications, but genomic imprints are sex specific, although most of these epigenetic control regions are matrilineal. Regardless of the parental origin, the imprinted gene is expressed or ***silenced*** in the same way in both male and female offspring. Some imprints are tissue specific but always show ***monoallelic expression*** (only one allele of a gene is actively transcribed). At this stage there is no evidence that the imprint is individually specific or that each father (or mother) has an individual "signature," but this is due more to the species studied to date (e.g., inbred laboratory rodents) than demonstrated as a general principle. It is known that if strains are crossed the imprint changes, but as yet there is no information on naturally occurring species and whether it is possible that the imprint may vary depending upon the population or perhaps lineage. This is a particularly important question in light of the role of ***sexual selection*** in the evolution of traits in outbred (versus inbred) species. For example, if male A mates with two females (E and G), *would the paternal imprint of the offspring of the litters (or singleton) produced from those matings be different or the same*? The converse question would apply to a female that produces two litters by different males (let us further assume that each litter has but one father of all the young in the litter rather than being a result of multiple paternity). *Is her maternal imprint identical in the respective litters*? While DNA methylation is clearly involved in ***genomic imprinting***, the signal for the imprint is not yet known.

This chapter will not deal with genomic imprinting further as there are excellent reviews relating genomic imprinting to brain and behavior available (e.g., Keverne & Curley, 2008; Keverne, 2009). Instead, I will focus hereinafter entirely on environmentally induced epigenetic modifications.

Finally, it is important to consider the issue of life stages. Individuals are particularly sensitive and vulnerable to environmentally induced epigenetic modifications during early life stages or in the period of transition from one stage to another. The time of maximal neuronal plasticity is in the earliest stages of life, beginning before birth and, in mammals, up to weaning. Although the individual's capacity to respond to environmental change or insult with heritable phenotypic variation at a later stage is possible, it is during this early period that hormones and genotype predispose an individual's responses to future experiences throughout the life cycle as well as its susceptibility to developing disorders (Gilbert & Epel, 2008; Bateson & Gluckman, 2011). Although most research has focused on the earliest life stages (fetus and neonate), another period of extreme vulnerability is the period surrounding ***adrenarche*** (the increase in activity of the adrenal glands just before puberty) and ***pubarche*** (the onset of puberty). It is during adolescence that the body (including the brain) is reshaped by hormones and the individual graduates from dependence to independence, assuming the properties of maturity. Stressors experienced during this period also have enduring effects, including neural remodeling, impaired learning and memory, and altered emotional behaviors in adulthood.

MOLECULAR VERSUS MOLAR EPIGENETICS

Investigators in the field of epigenetics come from one of two distinct lineages. This split history is similar to the origins of the modern study of animal behavior where European ethologists and American comparative psychologists differed in their approach to behavior, both in perspective and substance (see Chapter 2). Both molecular epigenetics and molar epigenetics share a common history, namely the sixteenth- and seventeenth-century debates of ***preformationism*** versus ***epigenesis***. The central question then was, and continues to be, how a fully integrated multicellular organism develops from a single cell (the fertilized egg). Preformationists believed that adult features were present fully formed in the egg and simply unfolded during growth; August Weissman belonged to this group and asserted that the eggs contained all of the elements (later known as genes) to determine the phenotype that would develop. Those believing in epigenesis held that traits emerge as a consequence of the progressive interaction of the constituent parts of the zygote with the environment in which it develops. Although others such as Charles Darwin and Jean-Baptiste Lamarck were believers in epigenesis, the pivotal role of the environment in the developmental process was first demonstrated empirically by Oscar Hertwig (1894) and subsequently by Richard Woltereck

(1909), whose early work on *Daphnia*, an organism that can reproduce asexually by cloning, demonstrated that genetically identical individuals would develop very different morphs depending upon their environment.

Molecular Epigenetics

Prior to the 1940s, the gene as the unit of heritable material was a theoretical concept without a physical identity. In 1942 Conrad Waddington proposed the term *epigenetics* as a conceptual model of how genes might interact with their environment and give rise to the phenotype (Waddington, 1942). It is in this sense that the term *epigenetics* is commonly used in molecular and developmental genetics today, namely, "the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms" (Holliday, 1990, p. 329). This relatively recent area of research focuses on processes such as DNA methylation (addition of a methyl group at specific positions on two of the four DNA bases) and histone modifications (changes to the proteins that package DNA) that are heritable in the short term but do not change the DNA or create mutations. Because artificial manipulation of methylation patterns is often lethal, or at the least results in ***maladaptive*** traits or monsters, this method of research illuminates normal development by creating abnormalities or anomalies. It also helps us to understand the processes that occur when environmental factors affect methylation of DNA during the normal development of organisms.

Waddington continues to dominate the way we think of epigenetics, and his image of an epigenetic landscape as an emergent process is the defining concept of how epigenetics operates. His structural depiction envisions how the environment shapes phenotypic outcomes and also the importance of timing as well of genes and environment. Although Waddington's formulation conveys the idea that development is irreversible and results in discrete outcomes rather than continuous outcomes, these aspects have now been refuted.

Attendant concepts advocated by Waddington (1942) were ***canalization*** and ***genetic assimilation***. Canalization connotes the differentiation of the gene, cell type, and embryo as development progresses and pathways becomes more entrenched, thereby making it harder for the canalized development to be dislodged and moved into another pathway. The concept of genetic assimilation emerged in part from his work with *Drosophila*. Waddington speculated that environmentally induced changes in phenotype could become incorporated into the genome, as evidenced by the persistence of the phenotype even after the original selection pressure is relaxed. It is in this manner that natural selection acts on developmental pathways leading to adaptive change in the genome rather than relying on genetic mutation. This dynamic view of

development incorporates both ***homeostasis*** (the stability of a final steady state) and ***homeorhesis*** (the stability of the process of development itself).

Molar Epigenetics

There are two types of molar epigenetics. The first arose from early evolutionists who asked how different phenotypes within a species were shaped by different environments. This area of study fell out of favor for about 60 to 70 years in European and American science. Interestingly, it continued as a major field of study in Russia and was represented in small part in this country in the work of Theodosius Dobzhansky and his students, most notably Richard C. Lewontin (2000). Today, it has reemerged as a vigorous area of research among evolutionary biologists and behavioral ecologists. New research on the origins of *polymorphisms* (multiple phenotypes in a single species) and ***polyphenisms*** (multiple phenotypes from a single genotype) has led to a concept now commonly referred to as ***phenotypic plasticity***, which is considered one of the driving forces in the relatively new union of developmental biologists with evolutionary biologists (***evo-devo***).

The other type of molar epigenetics has an equally old history. In psychology there has long been an interest in behavioral development or behavioral organization. Zing-Yang Kuo, who worked principally in the 1920s and 1930s, created much of the theory. Unfortunately, Kuo returned to China where the political strife interrupted research, and as a consequence his contributions were marginalized (Kuo, 1967; Greenberg, 2000). The other major figure in the field was Karl S. Lashley and his students, most notable for the purposes of this review Frank A. Beach (regarded as one of the founders of neuroendocrinology) and Theodore C. Schneirla (whose approach to the development and display of species-typical behaviors focused on the interaction of the genetic and the environmental levels of biological organization). Examples of this integrative approach are now numerous, but two classic efforts were those of Daniel S. Lehrman and Jay S. Rosenblatt. Lehrman conducted elegant work on the elaborate interaction of parent and offspring that results in ring doves (*Streptopelia risoria*) learning to care for their young, and Rosenblatt carried out exquisite research on the physiological and behavioral events that underlie the development of maternal behavior in cats and later rats. Both were students of Schneirla and emphasized the dynamic nature of a process that involves the interaction of the internal milieu and the organism and the interaction of the organism and its environment. Moreover, they defined the environment broadly to include the behavior and physiology of socially important species members. In so doing they laid the foundation for psychobiology, a vibrant field that focuses on how experiences accumulate

throughout life to shape the way in which the individual interacts with its social and physical environment (Gottlieb, 2002).

It is not my purpose to venture into the relatively unexplored frontier that lies in uniting the two subdisciplines of molar epigenetics (namely that of evolutionary and developmental biology and psychobiology) and behavioral neuroendocrinology. However, it is useful to be reminded of Ernst Mayr's constant refrain that behavior is at the leading edge of evolution and the observation of Michel and Moore (1995, p. 178) that "mechanisms that underlie much of behavioral evolution may reside in the processes studied by developmental psychobiologists."

However, it is necessary to emphasize before going further that the individual is the unit of selection and that an approach that integrates both molecular and molar epigenetics will be necessary to reveal the mechanisms that underlie behavioral evolution (Bateson & Gluckman, 2011). That is, the continuity between molecular and molar epigenetics is revealed as the constituent elements interact both positively and negatively in a temporal, spatial, and conditional (internal as well as in the social and physical environments) context (Nijhout, 2004). As adaptive responses emerge, they, in turn, set the stage for future variation. Thus, evolution is a tandem process involving first development, with its built-in flexible responsiveness to both gene products and environment, followed by selection, which dictates which variants are spread and maintained (Stearns, 1989; Lewontin, 2000; West-Eberhard, 2003). In this sense the "genome learns from its experience" (Jaenisch & Bird, 2003).

Obviously, suites of genes underlie the fundamental plasticity of an organism, particularly during development or life-stage transitions. *How do these gene networks interact with the experiences that accumulate during an individual's life history*? An important interface between the environment (either internal or external) and the genotype is that of epigenetic modifications. Exactly how these modifications come about is still relatively unknown, but recent studies at both the molecular and molar levels indicate that the origin of such effects may occur in previous generations. That is, experiences of earlier generations can modify regulatory factors affecting gene expression such that the DNA sequence itself is not changed but the individual's physiology and behavior are substantially influenced. Understanding how such modifications actually occur will increase our understanding of how the environment influences the relationship between genotype and behavior during sensitive developmental periods.

Before reviewing this literature, it is important to distinguish between mitotic versus meiotic epigenetic modifications, or what I have termed ***context-dependent*** versus ***germline-dependent*** epigenetic modifications (Crews, 2008, 2010).

CONTEXT-DEPENDENT VERSUS GERMLINE-DEPENDENT EPIGENETIC MODIFICATIONS

The scope of environmental effects that influence patterns of gene expression in the brain and subsequently behavior is virtually limitless. The extent to which environmentally induced epigenetic modifications can become inherited traits depends both upon the nature of the stimulus and the mechanism of its action. At a molecular level, CpG sites (regions of DNA where a cytosine nucleotide and a guanine nucleotide are adjacent) are often associated with 5' promoter regions of genes and have a higher probability of undergoing mutation than other regions of the genome. Consequent changes in DNA methylation patterns at CpG islands (DNA regions that contain a high frequency of CpG sites) would persist and, if imprinted in the germline, have the potential of becoming heritable.

Context-dependent Epigenetic Modification

Best studied are the epigenetic modifications that either have an effect early in life, such as exposure to ***endocrine disruptor chemicals*** (EDCs) in utero or smoking during childhood and adolescence. In the first instance the onset of disease manifests later during the individual's lifetime, while in the latter instance, the deleterious effects of smoking decline with time only if the individual is no longer exposed to the stimulus. Similarly, an epigenetic modification can be perpetuated across generations by simple persistence of the causal environmental factor such that each generation is exposed to the same conditions. For example, if the diet (Dolinoy, 2008; Faulk & Dolinoy, 2011) or environmental toxicant such as lead continues to be present in the environment, then the epigenetic modification will be manifested in each generation. This type of epigenetic modification lends itself to relatively straightforward therapeutic venues such as providing methyl donors in the diet (Dolinoy, 2008), and removing the environmental toxicant, whether smoking or lead. Hence, the environmental exposure will induce ***epialleles*** (genes that differ in the extent of methylation but otherwise are identical), but this environmentally induced epigenetic state can be reversed by a different environmental factor. This mitotically based effect can be termed "context-dependent" epigenetic change (Crews, 2008).

The best example of context-dependent epigenetic modification and behavior is that of Meaney and colleagues (Kappeler & Meaney, 2010). In a long series of elegant studies this group has demonstrated that the nature and amount of care a pup receives from the mother modulates its reaction to stress later in life, largely through effects on the glucocorticoid receptor (GR) in the hippocampus. This maternal effect can cross generations, but its heritability

depends upon the pup's experience in the first week of life. Recently this group has documented that being reared by a high-quality mother results in the expression of the transcription factor A (NGFI-A), a nerve growth factor–inducible protein, that binds to the first exon of the GR gene, resulting in increased expression of GR. High-quality maternal care during this critical period demethylates NGFI-A and the acetylation of histones. Just as cross-fostering can reverse these molecular and behavioral changes, infusion of methionine, a histone deacetylase inhibitor, into the hippocampus can also reverse these events. It is important to point out, however, that the effect of high- and low-quality mothering cannot be selected for and eventually disappears after five generations. That is, it is neither possible to selectively breed for quality of maternal behavior or to pass the effect nongenomically indefinitely.

Germline-dependent Epigenetic Modification

Germline-dependent epigenetic modifications are fundamentally different than context-dependent epigenetic modification in that the epigenetic imprint has become independent of the original causative agent. That is, the epigenetic modification is transferred to subsequent generations because the change in the epigenome has been incorporated into the germline. Thus, the effect is manifested each generation, even in the absence of the causative agent. In such instances the DNA methylation of heritable epialleles is passed through to subsequent generations rather than being erased as occurs normally during gametogenesis and shortly after fertilization. It is important to note that because germline-dependent epigenetic modifications are mediated through the germline, they tend to be sex linked. Examples of this type of epigenetic modification are still relatively rare. Nonetheless, the work of Skinner and collaborators on certain pesticides and fungicides demonstrates that such effects can occur (see below).

Transgenerational Inheritance

The defining distinction between context- and germline-dependent epigenetic modifications lies in the timing and persistence of the exposure. Exposure to environmental or psychological stressors will bring about change in the epigenome, but the transmission of the effects of that exposure can occur in two basic ways. Context-dependent epigenetic modifications are in direct response to the stimulus. Thus, an endocrine disruptor in the environment will induce changes in all individuals that are exposed to it and, as long as the environment stays contaminated, further generations will also exhibit

the modification (unless individuals undergo adaptive molecular changes that buffer them from the toxicant). All of the exposed individuals will have a body burden of the chemical and can pass that to their offspring (males can transmit the effect to their biological children or F1, whereas females can pass it to both their offspring and their grandchildren, or F2, but not to the F3 generation) (Skinner, 2008). On the other hand, germline-dependent epigenetic modifications can be transmitted to future generations without the requirement of additional exposure. In such instances removal of the contaminant will not result in resumption of the original, nonmodified state because the modification has become part of the germline and will pass to all future generations. Thus, only germline-dependent epigenetic modifications are truly transgenerational in nature.

Some might argue that using the term *epigenetics* without referring to a specific epigenetic mechanism is unacceptable. However, as detailed above, a proper definition of epigenetics extends beyond that used by molecular biologists, incorporating in addition functional outcomes. Thus, transcription, physiological, brain, and behavior changes all fall within the proper definition of epigenetics.

EPIGENETICS IS A PERSPECTIVE, NOT A TECHNIQUE

By now it should be obvious to the reader that the most important aspect of how one goes about studying behavioral epigenetics is to realize that it is more an issue of perspective or question and less of the tools and techniques to be implemented. For this reason virtually any aspect of behavior is open to the investigator; tissue differentiation, developmental psychobiology, cognitive development, psychopathology, life-history strategies, and phenotypic plasticity are just a few examples. After the question is formed comes the choice of organism to study. The two basic choices are naturally occurring species and conventional animal models. It is important to know the advantages and disadvantages of each. By studying diversity (naturally occurring species) we gain insights into evolutionary and ecological principles that can then be applied to vertebrates, including mammals (Crews & Moore, 1986). In my own work, discoveries made with lizards, such as hormone independence of sexual behavior and the role of progesterone and its synergy with testosterone in sexual behavior of males, have also been demonstrated in mammals and have become important fields of investigation. However, naturally occurring species have certain drawbacks, not the least of which is that they require special environmentally relevant cues not easily simulated in the laboratory. Another important disadvantage is the fact that usually only adult individuals are available

for study. Moreover, the investigator should always keep in mind that, compared with the original population, these individuals are only the ones that have managed to survive, and that the experiences they may have encountered as they grew are often lost to us. Also, many of the molecular tools that are routine to those working with rats and mice are not readily applied to the unconventional animals. Thus, those interested in the development of behavior, particularly how events early in life influence later behavior or how the epigenetic changes that occur from particular experiences may alter future behavior, may find it necessary to use conventional animal models. These animals have been stripped of their ecologically relevant traits, are well studied, and have been the template on which molecular tools were forged. Again the investigator must keep in mind the limitations of the animal. In general, model systems are analogous to a dragster. Basically, in drag racing the machine is an engine on a chassis guided by a driver, with the goal of getting to the quarter-mile mark as fast as possible. This is no different from an inbred strain of rodent, bird, amphibian, fly, nematode, or other organism in that inbred strains have maximized fitness (reproduction and growth rate) in an artificial and basically barren environment. That is, the conventional animal model organism is basically a gonad guided by a brain, with the "goal" of reproducing as fast as possible. In both the model organism and the dragster, there are no "bells and whistles" that may be demanded by the average customer, or in the case of a C57 mouse, a day in the life in the wild.

Next comes the question of what phenotype to study. A phenotype consists of multiple traits; each trait is defined as any measurable aspect of the individual. In general, our understanding of a particular phenotype increases proportionally with the number of traits that are measured in the same individual. Selection of the particular morphological, physiological, behavioral, and brain nucleus traits should be predicated on the literature and demonstrated to be important to the question at hand. The same principle applies to genes in that individual genes only have meaning in the context of other genes within and outside their functional categories.

EXAMPLES OF ENVIRONMENTAL FACTORS BRINGING ABOUT EPIGENETIC CHANGES

It is important to note that study of epigenetic modifications need not delve into the molecular underpinnings. The nature of the question is what is important, not the techniques employed. Several examples will illustrate this point.

Temperature and Sexual Experience as Agents of Epigenetic Modification

In the leopard gecko (*Eublepharis macularius*), temperature rather than sex chromosomes determine gonadal sex. Low (26°C) and high (34°C)

incubation temperatures produce only females, while intermediate incubation temperatures produce different sex ratios; 30°C (Tf) produces a female-biased sex ratio (25:75, or Tf), and 32.5°C a male-biased sex ratio (75:25, or Tm). Incubation temperature not only establishes the gonadal sex of the individual but also accounts for much of the within-sex variation observed in the morphology, growth, endocrine physiology, and aggressive and sexual behavior of the adult (Sakata & Crews, 2004). For example, males in general grow more rapidly and are larger than females from the same incubation temperature; Tm males, however, grow more rapidly and to a larger size than do Tf males. Hatchling, young, and adult Tm and Tf males do not differ in circulating concentrations of androgens. Estrogen levels do differ significantly, however, with Tf males having higher levels than do Tm males. Despite this similarity in circulating androgen levels in adulthood, males from the two temperature morphs differ significantly in their scent-marking response to exogenous hormones in adulthood, indicating neuroendocrine differences between the Tf and Tm males. There are also between-sex as well as within-sex differences in glucocorticoid levels in response to stress. Females have higher circulating levels of corticosterone than males, but, for both females and males, Tm individuals have significantly lower levels than do Tf individuals. Brain neurochemistry is also influenced by incubation temperature. For example, a significantly higher number of TH-ir (tyrosine hydroxylase immunoreactive) cells are found in the ventral tegmental area (VTA) of sexually inexperienced Tf versus Tm males that had been castrated and androgen-implanted, suggesting that embryonic temperature plays a role in differentially organizing dopaminergic (DA) systems of the temperature morphs. This is supported by the finding of significantly higher DA levels in the nucleus accumbens of Tf males compared to Tm males that have interacted with a receptive female across a barrier. Finally, sexually experienced Tf and Tm males both show strong preferences in a Y-maze apparatus to females or their odors, but the type of female they choose depends upon their incubation history. For example, given the simultaneous choice between two females from different incubation temperatures, Tf males prefer females from eggs incubated at high temperatures (34°C), while Tm males prefer the Tf females. Among females, Tm females are less attractive to males than are Tf females and will even attack males, a typically male pattern of aggression.

The long-term effects on the brain of significant life-history events are best revealed using cytochrome oxidase (CO) histochemistry. This is one of many measures of metabolic activity, but it has the advantage that CO is a rate-limiting enzyme in oxidative phosphorylation, the major pathway in brain metabolism. Consequently, the abundance and activity of CO activity in a

brain area is a measure of the metabolic capacity of that brain region over time. In other words, CO abundance not only reflects the metabolic history of an area but, because it determines the amount of ATP available in a neuron, it constrains the amount of activity a neuron can sustain (Sakata et al., 2005). It differs from other types of brain activity measures such as 2-deoxyglucose, immediate early gene expression, magnetic resonance imaging, and so forth in that it does not assess the current activity of the brain area so much as its past history of activation.

As in other vertebrates, the septum (SEP), ventromedial hypothalamus (VMH), anterior hypothalamus (AH), nucleus sphericus (SA) (homolog of mammalian medial amygdala), preoptic area (POA), and periventricular preoptic area (PP) are major integrative areas for hormonal effects on sexual and agonistic behavior in the leopard gecko. Incubation temperature influences the metabolic capacity of forebrain nuclei in adult leopard geckos, and, further, these differences correlate with the differences exhibited in their sexual and agonistic behaviors as adults. Sexual experience also influences the organization of the neural circuits underlying social and sexual behavior. As illustrated in Figure 8.1, the functional landscape changes significantly according to incubation temperature of the embryo but not so much according to the sexual experience of the individual in adulthood. That is, if one examines the effect of incubation temperature (comparison within columns), both inexperienced and experienced Tm females show greater activity in the AH, NS, and SEP (but not in the POA, VMH, or PP, which are unchanged). The POA and AH particular nuclei are centrally involved in maturation of the hypothalamus-pituitary-gonadal axis and the NS and SEP in the maturation of the hypothalamus-pituitary-adrenal axis. A different picture emerges when comparing inexperienced and experienced females from within each incubation temperature (comparison within rows). In this instance adult sexual experience modifies the effect of embryonic incubation temperature in the POA in the all-female incubation temperature and in the POA and VMH in Tm females, but to a lesser degree (note difference in scale). This suggests that in the leopard gecko incubation temperature has a more profound effect on brain organization than does adult sexual experience.

Litter Composition Shapes the Development of Brain and Behavior

For those interested in behavioral development in mammals, it is not necessary to go further than the litter environment for the context in which epigenetic effects can occur. Specifically, it is precisely this period of postnatal development, during which the individual is nurtured within the litter, that most influences its behavior as an adult and, as such, the activity of the neural

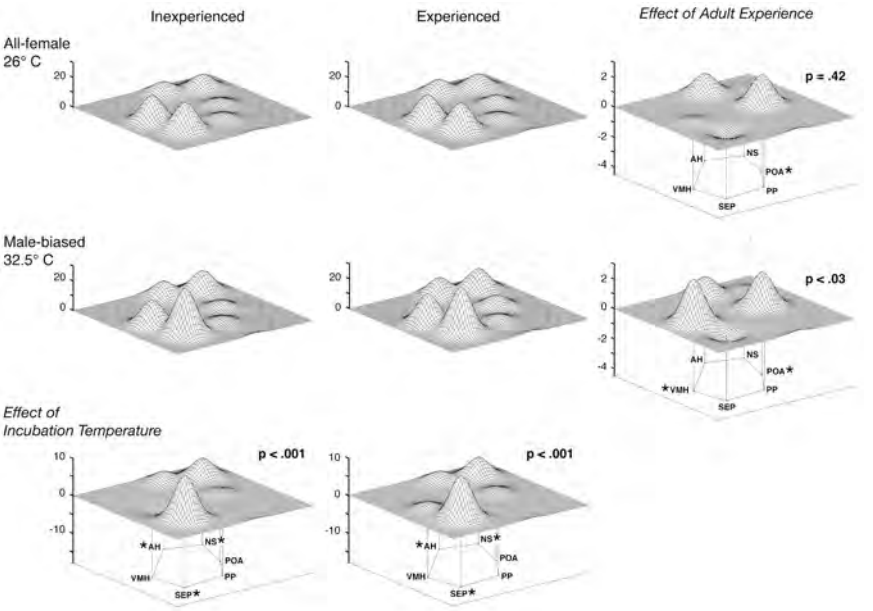

Figure 8.1. Incubation temperature modifies the abundance of cytochrome oxidase in limbic nuclei subserving sociosexual behavior in the adult female leopard gecko (*Eublepharis macularius*). Illustrated are means of cytochrome oxidase abundance relative to background in each nucleus of each of four groups of geckos. Eggs were incubated at one of two temperatures (all-female, or 26°C, and male-niased, or 32.5°C), but the hatchlings were raised at identical temperatures. At one year of age females were allowed to breed for one reproductive season (experienced) or remained inexperienced. Note that in both sexually inexperienced and experienced groups (columns), comparison of the two different incubation temperatures results in a significant percentage increase in most, but not all, nuclei relative to overall brain activity. This in turn results in significant differences in the overall circuit. Within each incubation temperature (rows), however, adult sexual experience modifies the effect of embryonic incubation temperature in only the POA (all-female) or the POA and VMH (male-biased) nuclei. The effect of experience on the overall circuit is not significant at the all-female temperature and only marginally significant at the male-biased temperature. Values are average cytochrome oxidase abundance in identified cell nuclei relative to background. Brain nuclei: ventromedial hypothalamus (VMH); anterior hypothalamus (AH); nucleus sphericus (NS); preoptic area (POA); periventricular preoptic area (PP); septum (SEP). The bottom row reveals the effect of embryonic temperature; peaks above the plane indicate values that are greater at the male-biased incubation temperature. The right column reveals the effect of adult experience; peaks above the plane indicate values that are greater in sexually experienced individuals. An asterisk indicates significant differences in particular nuclei.

circuitry that underlies these behaviors. Consideration of such epigenetic effects is particularly needed in studies of genetically modified mice, on which much of the genotype × environment (interaction of genotype and environment) research is conducted. Although the mouse is ideal for genetic work, too often the investigators expediently will assign animals to experimental groups without considering family-of-origin issues such as sex ratio, genotype ratio, size of litter, maternal care, or unanticipated stresses that occur in any colony. These issues are not trivial, as any behavioral neuroscientist working with rats knows, but may not be considered important by molecular neuroscientists who work principally with mice. It might be argued, as a justification for this research flaw, that in the colony as a whole there exists a Mendelian genotype ratio and an even (50:50) sex ratio. However, this is not a valid argument because the experimental animals actually used in the study do not come from a population but from a litter that has a particular sex and genotype ratio. In model systems that are the result of the mating of heterozygotes (HTZ) to yield litters of varying numbers of wild-type (WT), HTZ, and ***knockout*** (KO) (genetically engineered mouse with an inactivated or "knocked-out" gene) young of both sexes, the ratio of the various genotypes is as important as the sex ratio of the litter. Appropriately designed studies need to control for the distribution of individuals within the groups representing all litter types. Indeed, until the investigator can show that both sex ratio and genotype ratios are equally distributed and litters are equally represented in all of the experimental groups, any conclusions are suspect.

This is a bold statement, but evidence backs this contention. In collaboration with Sonoko Ogawa, I have examined how the sex and genotype ratios of a litter might contribute to the development of behavior in mice having a null mutation of the estrogen receptor αERα (Crews et al., 2004, 2009). By mating mice heterozygous for a null mutation for this gene it is possible to reconstitute litters shortly after birth to control for both sex (♀/♂) ratio and KO and WT genotype ratios. Thus, the possible combinations are (1) same-sex, same-genotype litters (e.g., ♀WT/♀WT); (2) same-sex, mixed-genotype litters (e.g., ♀WT/♀KO); (3) mixed-sex, same-genotype litters (e.g., ♀WT/♂WT); or (4) mixed-sex, mixed-genotype litters(e.g., ♂KO/♀WT). By reconstituting the litters into one of the 16 possible combinations, the effect of genotype can be examined without the potential confound of the presence of the opposite sex in the litter, and the effect of siblings of the opposite sex can be studied without the potential confound of littermates with a different genotype.

The results of such studies indicate clearly that both factors are important in shaping the behavior when the individual is an adult. For example, one of the behavioral diagnostics of ERαKO female mice is that as adults they are

very aggressive (Ogawa et al., 1998). However, this occurs only if ♀KO are raised in litters containing other ♀KO; if raised with ♀WT or ♂WT they fail to show aggressive behavior and are comparable to ♀WT mice in social contact time. Indeed, litter composition influences the development of sociosexual behaviors in ERKO mice of both sexes. Extending this work to the brain, I examined the pattern of metabolic activity in various brain nuclei of the mice raised in these controlled litter groups (Crews et al., 2009).

It is of interest that WT females raised in same-sex, same-genotype groups spend significantly more time in social contact in a resident-intruder test compared to KO females raised in same-sex, same-genotype groups (Figure 8.2). Further, it appears that female WT siblings are able to compensate for this deficit, just as KO siblings cause a deficit in WT females. The neural network

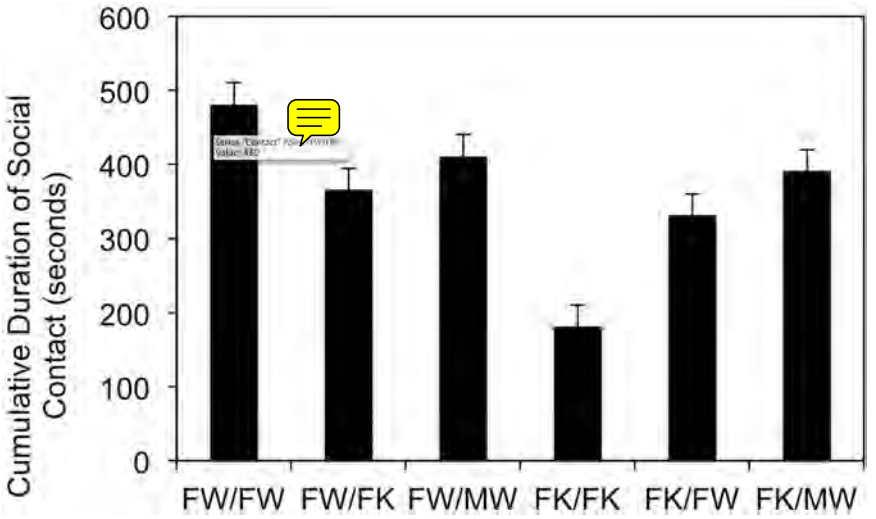

Figure 8.2. Social contact time in genetically modified mice raised in reconstituted litters arising from mating of mice heterozygous for a null mutation of the estrogen receptor a gene. Litters consisted of equal numbers of wild-type (WT) or knockout (KO) female (F) or male (M) mice. Shown are results of individuals raised in single-sex, single-genotype litters (FW/FW and FK/FK); single-sex, mixed-genotype litters (FW/FK and FK/FW); mixed-sex, single-genotype litters (FW/MW); and mixed-sex, mixed-genotype litters (FK/MW); The tested animals were the sex and genotype of the first symbol (e.g., a female WT individual raised in the single-sex, mixed-genotype litter (FK/FW). Significant differences occurred between FW/FW and FK/FW, FW/- and FK/FK, and FK/FK and FK/MW.

that underlies sociosexual behavior varies in different ways. The relative effects of sex independent of genotype, and of genotype independent of sex, on the neural network are striking (Figure 8.3). Taken together these findings indicate that in studies with genetically modified mice, litter composition during the preweaning period must be considered because it can affect the development of behavior and the neural network responsible for the regulation of emotional behaviors.

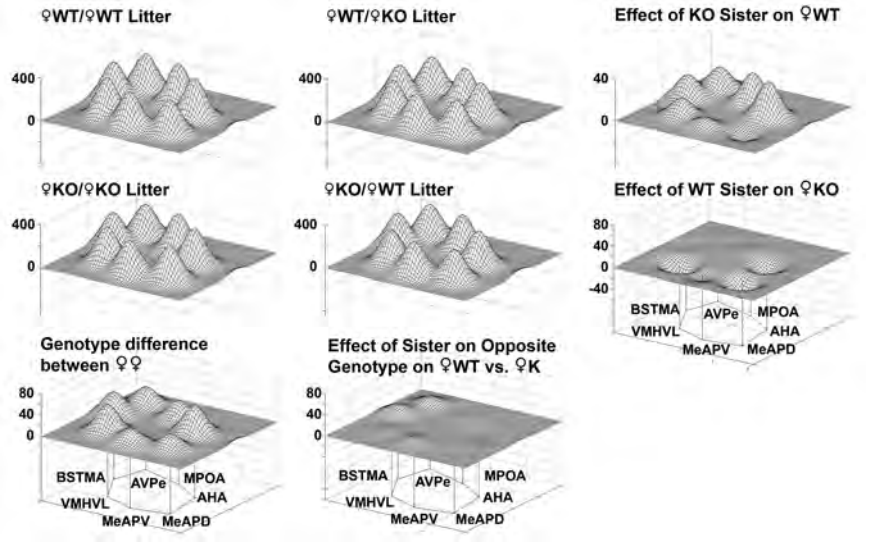

Figure 8.3. Effect of genotype of sisters on the metabolic activity in a social behavior network in female wild-type (WT) and estrogen receptor α knockout (KO) mice. Groups are presented according to the type of sibling with which the WT (top row) or KO female (middle row) was raised, with ♀KO or ♀WT sisters, respectively. The limbic functional landscape map on the upper right represents the difference in ♀WT raised with a ♀KO sister. Below that is the complement, that is, the effect of a WT sister on the metabolic activity in ♀KO females. The bottom row indicates genotype differences between ♀WT and ♀KO females raised in same-sex, same-genotype litters. The far right column shows the effect of having a sister having the opposite genotype. The nuclei are presented in a clockwise fashion reflecting a rostral-caudal dimension: main bed nucleus of the stria terminalis (BNSTma); anteroventral periventricular nucleus (AVPe); medial preoptic area (MPOA); anterior hypothalamus, anterior (AHA); medial amygdaloid nucleus, posterodorsal (MeAPD); medial amygdaloid nucleus, posteroventral (MeAPV); and ventromedial hypothalamic nucleus, ventrolateral (VMHVL).

Endocrine Disrupting Chemicals and Epigenetics

Whether intentional or not, an array of chemicals that mimic or block the action of endogenous hormones are now a permanent part of our environment. Classified as EDCs, they behave as biological signals and activate the parts of the endocrine system associated with the steroid/retinoid/thyroid superfamily of receptors (McLachlan, 2001). Exposure can be limited to a very restricted period, exist throughout the individual's lifetime, or have occurred only in previous generations. Any or all of these exposures can influence all aspects of an individual's life history.

It is becoming evident that the mechanism of action of EDCs is probably epigenetic—in other words, they cause heritable changes in gene function without changing the DNA sequence, that is, without causing mutations (these are sometimes referred to as ***epimutations***) (Crews & McLachlan, 2006). Michael Skinner and his group (Skinner & Guerrero-Bosagna, 2009; Skinner, 2011) provided conclusive evidence that EDCs can reprogram methylation patterns in the germline, and hence their effects can be transmitted to future generations and expressed without further exposure. They demonstrated that exposure of gestating female rats to the pesticide methoxychlor or the fungicide vinclozolin during the period of embryonic sex determination induces an epigenetic transgenerational phenotype through reprogramming the germline in a sex-specific manner. Specifically, in each generation males whose ancestor had been treated underwent progressive spermatogonial apoptosis (cell death), decreased sperm count and motility, and, as the animals aged, an accelerated development of adult-onset disease including cancer, prostate disease, kidney disease, and immune-cell defects. A series of new imprinted-like genes that transgenerationally transmits this altered epigenome to promote disease phenotypes appears not only in the sperm epigenome but also in the brain epigenome (Skinner et al., 2008).

The behavior of these individuals is also epigenetically modified. Females discriminate and prefer male descendants of the line that was not exposed to the chemical, whereas similarly epigenetically imprinted males do not exhibit such a preference (Crews et al., 2007) (Figure 8.4).

Specifically, in a partner-preference test, F3-generation females of both the vinclozolin and control lineages discriminate and prefer males who do not have a history of exposure; males do not exhibit such a preference. Odor-preference tests rule out possible differences in the odor-discrimination ability of epigenetically modified animals; 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.

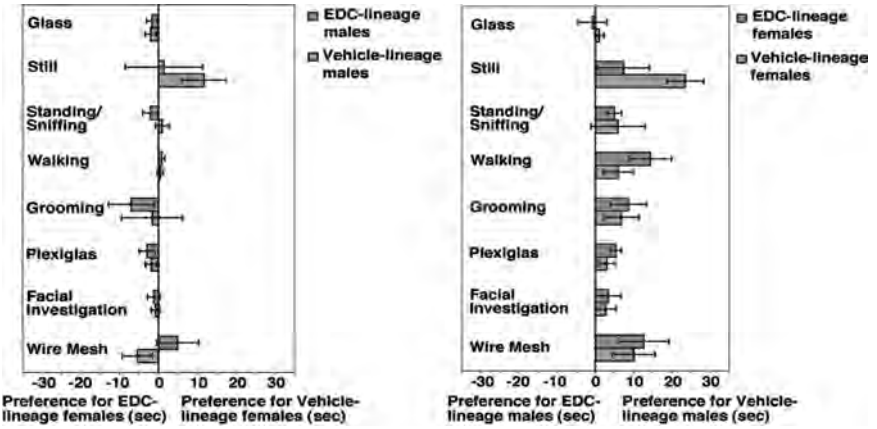

Figure 8.4. Female rats whose great-grandmothers were exposed to the EDC vinclozolin, a commonly used fungicide with endocrine-disrupting properties, and hence were epigenetically altered, prefer males from the unexposed vehicle lineage (right panel). Males do not show this preference (left panel). Both females and males from control and EDC lineages were tested with pairs of vehicle- and EDC-lineage stimulus partners. Presented are the mean (+1 standard error) differences in the time spent in each behavior. Right panel: behaviors exhibited by females from vehicle and EDC lineages towards males from vehicle lineage (positive, right side) and EDC lineage (negative, left side). Left panel: behaviors exhibited by males from vehicle and EDC lineages towards females from vehicle lineage (positive, right side) and EDC lineage (negative, left side). Data from Crews and colleagues (2007).

Stress Induces Context-dependent Epigenetic Modifications

Stress, particularly if sustained, can lead to impaired immunity, disease, and neurological changes characteristic of major depressive illness and particularly chronic anxiety disorders (Romeo et al., 2009; McEwen, 2010). Chronic restraint stress (CRS) in rats has been a standard paradigm for studying such effects on physiology, brain, and behavior. For example, six hours daily of immobilization restraint for three weeks results initially in elevated corticosterone levels, but after 21 days, the hypothalamic-pituitary-adrenal axis shows adaptation and levels are back to normal. This, however, underlies a progressive atrophy of the dendrite length and branching of pyramidal neurons in the CA3 region of the hippocampus, a process mediated by corticosterone potentiating postsynaptic activity and the release of excitatory amino acids from adjacent mossy fiber terminals arising from the granule neurons in the dentate gyrus and acting via NMDA (glutamate) receptors. Conversely, there is an increase in dendritic spine density of neurons in the basolateral amygdala

and medial prefrontal cortex (mPFC) and decreased neurogenesis in the dentate gyrus. In addition to these structural changes, stressed rats exhibit a variety of specific cognitive deficits in spatial learning and memory, as well as increased anxiety-like and agonistic behavior.

The effects of stress, however, appear to vary depending upon the sex of in the individual and when the stress occur (Shors, 2006; Romeo et al., 2009). For the purposes of this chapter, I will only consider the literature on male rats, as that is the epigenetic model best studied. In male rats the effects of chronic stress early in development tend to be irreversible, resulting in permanent structural changes in the hippocampus and altered adult sociosexual and anxiety-related behaviors, while those experienced as an adult can be reversed. If the stress occurs during the peripubertal-juvenile transition, the effects are similar to early effects, if not exaggerated. In rats, CRS influences serotonin and dopamine activity in CA3 of the hippocampus, dopamine and its metabolites in CA1 of the hippocampus as well as the mPFC, and dopamine and its metabolites in the basolateral amygdala.

Integration of Germline- and Context-dependent Epigenetic Modifications

It is clear that individuals modify, or even recreate, their environment via behavior. Two challenges that must be considered are (1) environmental stressors and (2) psychological stressors. Both are forced upon the individual, because they either are part of the environment the organism is born into or are visited upon it during its life. Both induce epigenetic modifications, but of a different sort. The former is dependent upon exposure while the latter can result in alterations to the genome of future generations independently of changes to the DNA sequence through mechanisms that include DNA methylation. There is now clear evidence that an individual's likelihood of developing health problems involves a combination of that individual's own exposures as well as exposures of ancestors in generations past. Another factor that must be considered is (3) the hormonally induced differentiation of body and brain triggered by genes, resulting in sex differences in physiological and behavioral responsiveness to environmental stimuli. In rodents and humans, males and females differ substantially in reactions to environmental challenges and their propensity to develop illness, disease, and affective disorders.

Recent studies with rats have combined both the germline-dependent (transgenerational epigenetic modification) effects with a context-dependent (CRS during adolescence) effect. In this case only males were studied, but the results clearly confirmed that a single exposure to vinclozolin three generations removed alters the brain epigenome, transcriptome, physiology, behavior, and metabolic activity in discrete brain nuclei in F3 descendant males.

What is also evident is that this transgenerational epigenetic modification causes the F3 males to respond differently to stress. The following are a few examples of such changes at the different levels of biological organization (Crews, 2011).

The pattern of body weight (BW) gain differs according to lineage and stress, with vinclozolin-lineage males gaining weight more rapidly and becoming heavier than control-lineage males; CRS abolishes this difference. In males, CRS results in lower corticosterone (CORT) levels in both the control- and vinclozolin-lineage groups. Lineage, but not stress, influences circulating testosterone (TESTO) levels. Among stressed animals, TESTO levels are significantly higher in vinclozolin-lineage males relative to control-lineage males.

At the level of behavior, a number of differences are evident (Table 8.1). The open-field (OF) test measures anxiety and emotionality. We find that control-lineage nonstressed (CL-NS) males spend more time in the corners of the OF than do vinclozolin-lineage nonstressed (VL-NS) males (Table 8.1). Exposure to CRS during adolescence has opposite effects in the two lineages; CL males move out of corners and into the center, indicating greater exploration, whereas VL males move from the center into corners, indicating greater anxiety (interaction between lineage and stress). There is also an effect of stress independent of lineage: stressed males move faster through the center than do nonstressed males, indicating that CRS during adolescence increases anxiety later in adulthood. Sociability 1 measures social approach, anxiety, and exploration. We find that lineage effects are restricted to the stress condition, with VL males traveling further and faster than the CL males and choosing to associate with the stimulus animal more than nonstressed individuals. In general, CRS during adolescence affects line crossing and latency to first entry into the chamber containing the stimulus animal. In the nonstress condition, VL males visit the stimulus animal for longer periods and move between chambers less than do CL males. Sociability 2 measures social novelty and working memory. We find that CL-NS males spend more time with the stimulus male that is novel than with the familiar male. Only VL males show effects of stress, traveling further and faster than VL-NS males; they also spend less time in the center compartment and more time with the familiar and novel stimulus males. Comparison of the two tests reveal that in Sociability 1, VL-S males tend to spend less time in the center compartment than do VL-NS males, a difference that becomes significant in Sociability 2, suggesting that following CRS, VL males display greater affiliation behavior with the familiar individual. In CL males, there is no effect of CRS, but mean center time decreases in Sociability 2, a difference significant only in the stress condition. Similarly, VL-S males tend to spend more time in the animal chamber in

Sociability 1 than do VL-NS males; in Sociability 2, this difference becomes significant, again suggesting formation of a social bond with the familiar animal.

At the level of brain nuclei, we find effects of both lineage and stress. Table 8.1 summarizes these findings, and Table 8.2 shows the nature of the effects. For example, amygdaloid nuclei are differentially affected by lineage; CO activity in posteromedial cortical amygdala (PMCo) is higher in CL males regardless

Table 8.1. Summary of data obtained at three different levels of biological organization in pilot experiment on the interaction of transgenerational epigenetic modifications three generations removed and exposure of chronic restraint stress during adolescence on adult behavior of male rats. VL = vinclozolin lineage; CL = control lineage; S (stress) = restraint stress during adolescence; NS = nonstress; L×S Interaction = interaction between ancestral and proximate exposures; Yes = an interaction exists; No = no interaction exists. Symbols: – = no effect; > = greater in one group compared to the other; ++ = statistically significant difference; – = no statistically significant effects.

Trait	**Lineage**	**Stress**	**L×S interation**
Body weight	VL > CL	++ (VL)	Yes
ASI	VL > CL	++ (VL)	Yes
Corticosterone	–	++ (VL)	Yes
GSI	–	++ (VL)	No
Testosterone	–	++ (VL)	Yes
Leptin	–	–	No
Behavior	**Lineage**	**Stress**	**L × S interaction**
Forced Swim	–	–	–
Open Field	++ (CL)	++ (VL CL)	Yes
Sociability 1	–	trend (VL)	NS
Sociability 2	++ (VL)	++ (VL)	Yes
Nucleus	**Lineage**	**Stress**	**L × S interaction**
BLA	++ (S)	–	Yes
CA1	++ (S)	++ (VL)	Yes
CA3	++ (S)	++ (VL)	Yes
MeAmy	++ (NS)	++ (CL)	Yes
MePD	++ (S)	++ (VL CL)	Yes
PMCo	++ (NS S)	–	No
Stria	– (S)	++ (VL)	Yes

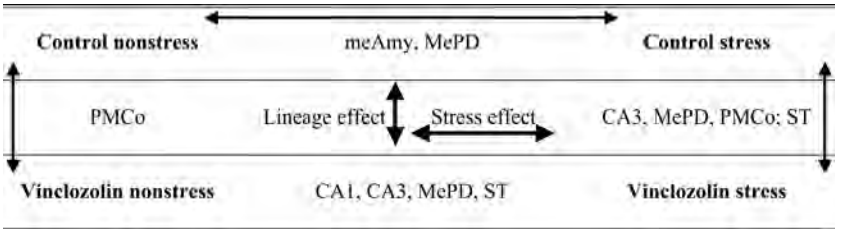

Table 8.2. Significant changes in cytochrome oxidase abundance in brain nuclei as a consequence of Lineage and Stress.

of stress condition. The medial posterior dorsal amygdala (MePD) shows opposite effects following CRS (pronounced increase in CL and decrease in VL males). In the medial amygdala (MeAmy), CO activity is opposite in the lineages depending upon stress. We also see that the stria terminalis (ST), and not the bed nucleus of the stria terminalis (BnST), shows marked changes, indicating that activity in this major pathway is being modified by lineage. In the CA1 and CA3 of the hippocampus, stress decreases metabolic activity in the VL males but has no effect in CL males.

Thus, the alteration of baseline brain development brought about by transgenerational epigenetic modification promotes a change in neural genomic activity that correlates with changes in physiology and behavior, revealing the interaction of genetics, environmental epigenetics, and epigenetic transgenerational inheritance in shaping the adult phenotype. This is the first empirical evidence in an animal that ancestral exposure to a known EDC modifies how descendants of these progenitor individuals perceive and respond to a stress challenge experienced during their own life history.

SUMMARY

We are at the very beginning of studies of the epigenetics of behavior. Behavioral phenotypes are affected by multiple factors, some beginning in generations past and others originating during sensitive periods or life stages. We now know that genes do not cause behavior, and there is little evidence that, outside of disease and pathology, genotypes predispose individuals to behave in particular ways. Understanding the development of behavior has yielded more information on the causes of behavior. For example, different experiences during sensitive life stages produce variation among individuals that markedly influence how the individual responds to social and sexual cues later in adulthood. This variation is the substrate on which evolution can act.

Several studies that deconstruct various confounds inherent in research in developmental psychobiology have been presented. For example, in the ERαKO mouse, we have seen how the sex and genotype ratios of the litter have separate and distinct effects on the nature and quality of the individual's behavior later in adulthood, as well as on the metabolic activity within networks of brain nuclei that underlie these behaviors. The finding that functional neural systems can be reorganized depending upon the composition of the litter in which the individual develops is startling yet yields a deeper understanding of how neural systems are organized early in life.

The recent discovery that the environment can affect the genome of future generations without changing the DNA sequence has particular relevance to understanding the brain and behavior. However, it remains to be seen if our increasing understanding of the causes and effects of epigenetic modifications will produce generalizable insights into the causes and functions of behavior. This chapter describes some of the mechanisms by which factors influence adult behavioral responsiveness and their underlying neural substrates; of particular interest in this regard is how the environment can produce significant individual variation in social behaviors. Two distinct epigenetic modifications are described: context-dependent modifications are similar to proximate environmental effects, while germline-dependent modifications are equivalent to ultimate environmental effects in shaping brain and behavior.

It is clear that ultimate and proximate events interact to influence how an individual responds to events in its own life history, and the study of epigenetics may be the method by which these issues can be addressed. To date, only one study has demonstrated that germline-dependent epigenetic modifications laid down in previous generations alter how individuals respond to a stressor (CRS) experienced during adolescence (Crews et al., 2011). This result suggests that different types of experiences can result in different epigenetic modifications that are independent of one another but that together influence the phenotype in novel ways.

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