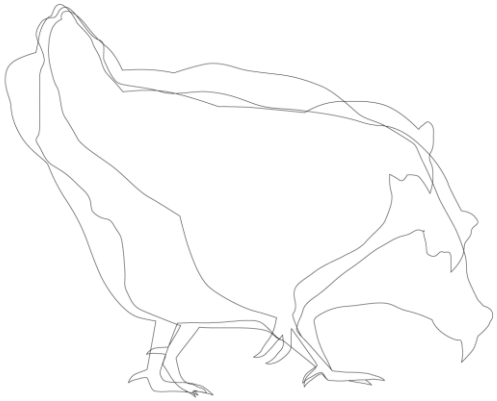
Behavioral Epigenetics and the Effects of Domestication

Introductory essay, 2013

Johan Bélteky

Supervisor: Per Jensen, Linköping University



AVIAN
Behavioural Genomics and Physiology group

Department of Physics, Chemistry and Biology, Linköping University, SE-581 83 Linköping, Sweden

Abstract

Rapid evolution and adaptation require mechanisms that can orchestrate genomic output and cope with the swift environmental changes and selection. Domestication is a clear example of an artificial selection that in a short time has given rise to a plethora of phenotypic variation, something that cannot be accounted for by sporadic mutations in the genomes of selected animals. A suggested explanation lies in epigenetics, the field of genetics that involve modifications of DNA and chromatin in order to regulate genomic output. Methylation of DNA is by far the most extensively studied of these modifications and a huge area of research in medicine, evolutionary genetics and behavioural sciences, just to mention a few areas. Along with the important histone modifications methylations and acetylations, these epigenetic markers lay way for alternative and dynamic regulation of gene expression. With more and more research being done on the subject, it is clear that epigenetic mechanisms are connected, and DNA methylations variations correlate with histone modifications in order to alter chromatin states. Albeit dynamic in their nature, the marks persistency change depending on the context of their use; some marks fade very rapidly whilst others may last a lifetime, or more, if passed through the germline to the offspring. With that possibility, the progeny might be programmed for certain nutritional conditions, or behavioral responses to various stimuli.

Keywords: Behavioral epigenetics, DNA methylation, domestication, epialleles, histone modifications, stress, transgenerational inheritance

Content

1 The process of domestication

Domestication, the process where animals are artificially selected upon to adapt them towards their captive environment and reinforce desired phenotypes, is in short an accelerated evolution (Price, 1999). More specifically, domestication has been defined as ‘‘that process by which a population of animals becomes adapted to man and to the captive environment by genetic changes occurring over generations and environmentally induced developmental events reoccurring during each generation’’ (Price and King, 1968, cited by Price, 1999). This phenomenon is not anything new. Studies on wolves, the ancestor of our common dogs, place the event of its domestication somewhere in East Asia, around 15000 years ago (Savolainen et al., 2002). Genetic analyses of mitochondrial DNA indicate that several wolf haplotypes exist, suggesting that the domestication of wolf was not a single isolated event but occurred simultaneously at several locations, making it events and not a single event (Savolainen et al., 2002; Vila et al., 1999). The same conclusion can be drawn when investigating the origin of our common fowl, the chicken (*Gallus gallus*). Chicken were domesticated from the red Junglefowl (RJF) around 8000 years ago in Southeast Asia, and via China spread to the rest of the world (West and Zhou, 1988). More recent research does however imply a somewhat mixed origin. Investigating the skin color of their legs, researchers have found that the grey Junglefowl has contributed with at least a small part of the genome to today’s breeds, based on the occurrence of the *yellow skin* gene in our domestic breeds, a gene originating from the grey Junglefowl (Eriksson et al., 2008).

The artificial selection that is domestication, is as explained a phenotypic evolution, and has been studied as such. A well known study is of the silver fox (*Vulpes vulpes*), a set of experiments started in the late 1950s by Russian geneticist Dmitry Belyaev (Trut, 1999). Belyaev figured that the behavior is at least partly determined by the animal’s genes and could therefore be bred on under a strong selective pressure, and set out to breed on tameness, and tameness alone. He suggested that selecting against aggression towards humans and only selecting on the animals willing to make contact with humans would in time bring out behaviors similar to those in dogs. Subtle changes indicating domestication, such as tail-wagging, arose in a few generations, and as of recently,

almost 40 generations later, a variety of behavioral, morphological and physiological changes can be seen in the animals (Trut et al., 2009). Alterations as these are named the domestic phenotype, and refer to the phenotypic changes that allow an animal to adapt to both man and the animals confined environment (Price, 1984). Just as in other domesticated animals, the silver foxes of Novosibirsk today exhibit curly hairs and tail, floppy ears, the appearance of dwarfism or gigantism, changes in body size and proportions, alterations of reproductive patterns to enable breeding at any time of the year, and most interestingly, neuroendocrine changes to the HPA axis. These changes demonstrate the correlation between traits, and how selections for a single one can affect so many others, such as the susceptibility to stress. With the new environment and stimuli that man provides captive animals with, a possible hypothesis is that the domestic phenotype is a result of a change in stress sensitivity needed for domestication.

2 Stress and its effects

When referring to stress, one usually means something that disrupts the homeostasis in the body, with a more narrow definition being suggested by Koolhaas et al. (2011): "[…] conditions and stimuli where predictability and controllability are at stake; unpredictability being characterized by the absence of an anticipatory response and loss of control being reflected by a delayed recovery of the response and the presence of a typical neuroendocrine profile". Biologically, stress affects the sympathetic nervous system (SNS), and is transmitted throughout the hypothalamic-pituitary-adrenal axis or HPA-axis for short. This neuroendocrine system is amongst other things involved in mediating signals relating to digestion, mood, the immune system and energy storage. Starting from "the top", the hypothalamus is responsible for releasing vasopressin (AVP) and corticotropin-releasing hormone (CRH), two peptides that in turn act on the pituitary, making it release adrenocorticotropic hormone (ACTH, also known as corticotropin). ACTH stimulates the adrenal glands to release glucocorticoids (GCs), such as cortisol/corticosterone, all within a few minutes after the initial stimulus on the hypothalamus. The glucocorticoids will then in turn act in a negative feedback loop and make the hypothalamus and pituitary seize endocrine production.

Catecholamines, more specifically dopamine (DA, precursor to noradrenalin), adrenalin and noradrenalin (NA) are neurotransmitters which are involved in physiological processes such as regulation of behavior and coping strategies to stressful conditions. Aggressiveness is generally correlated with higher concentrations of adrenalin, whilst high levels of DA can lead to an inhibition on the reproductive systems development and hyperactivity (Gainetdinov et al., 1999; Sgoifo et al., 1996). Another monoamine neurotransmitter, albeit not a catecholamine, is serotonin. Serotonin (or 5-hydroxytryptamine, 5-HT) is a substance with a broad spectra of actions within the CNS, both on physiological systems and behavioral functions such as aggression, mood, sleep, learning, sexual behavior and also response to stress (Higley et al., 1996; Lucki, 1998). Inhibition of 5-HT uptake carriers, as well as activation of 5-HT receptors by 5-HT agonists have both been shown to act on the HPA-axis, increasing serum corticosterone and ACTH (Fuller, 1995). Lowered transcriptional activity of 5-HT transporters have been shown to correlate with susceptibility to develop depression, and the heightened 5-HT baseline in domesticated foxes is thought to correlate with their reduced aggressiveness (Caspi et al., 2003; Trut, 1999).

Corticosterone, part of the HPA-axis and released from the adrenal cortex, is another stress hormone. Greater corticosterone production capability enables a more adaptive response to stresses, and GCs may also work as an immunomodulator (Cheng and Muir, 2005; Cheng et al., 2001). Higher corticosterone levels in White Leghorn have been linked to higher cellular-modulated immunity, whilst more aggressive birds had higher amounts of immunoglobulin G and circulating heterophil:lymphocyte ratio, a physiological indicator of stress (Cheng et al., 2001). In rats, increased handling, or maternal care, increases the hippocampal GC receptor gene expression which in turn regulates the HPA-axis via negative feedback in order to decrease AVP, CRH and corticosterone synthesis during times of stress (Liu, 1997). This in turn manifests as dampened response to stress, a plasticity derived from the mother's behavior and interpretation of the surrounding environment, and these responses to stress can be formed during the early postnatal period. Trut et al. (2009) also report dampening of the HPA-axis in the domesticated silver foxes, correlating with calmer behavior and seemingly an effect of the selection only on tameness.

Responses to stimuli can be categorized into coping styles (reviewed by Koolhaas et al., 1999). High sympathetic reactivity (high NA) is part of a proactive coping style (aggressiveness), whilst high HPA-axis reactivity (high corticosterone) is part of a reactive coping style. One example is feather pecking in White Leghorn chicken, in which high peckers showed a significantly higher NA response during constraint stress, whilst the low peckers displayed an increase in corticosterone during both restraint and rest (Korte et al., 1997). Neither group did show any differences in adrenalin response. Coping styles is an indication of what neuroendocrine and physiological mechanism are at use, and as such behaviors could for example indicate whether mechanisms in selected lines has been altered.

Stress can come in a variety of forms, such as heat stress, confinement or housing conditions with a large number of individuals. In a study on domesticated fowl, commercial broilers were found to be more susceptible to heat stress compared to RJF, when exposed to 36 degrees for 3h (Soleimani et al., 2011). The stressor affected plasma corticosterone levels, heat shock protein 70 and body temperature in the broilers. Even with a two-fold increase of corticosterone in broilers, the levels were still only half that of the unaffected RJF. Group selection in chicken with selection on productivity has the side-effect of decreasing both well-being and productivity (reviewed by Cheng and Muir, 2005). Differences in DA, adrenalin, NA and corticosterone can all be found between "good" (high egg production and longevity) and "bad" (high mortality, low production) groups of birds, and as mentioned previously different hormonal levels alter the adaptive possibilities for the individual. In summary, "bad" birds have elevated DA and adrenalin levels, and "good" birds have heavier adrenal glands which in turn suggest a higher adrenal activity.

Housing of birds in big groups has shown to be a stress with negative impact not only on the birds in the big housing groups, but also on the condition of their offspring (Naguib and Gil, 2005). Zebra finches raised in crowded environments show lower reproductive success, and a sex-specific link was found where daughters were much more affected (Naguib et al., 2006). Large broods and the stress it inflicts affect factors such as body size, and similar results have been found when investigating nutritional stress (Krause et al., 2009; Naguib et al., 2006). A final example is a study by Lindqvist

et al. (2007), which found that early-life stress by disruption of circadian rhythm causes behavioral and gene expression changes in White Leghorn, factors that are transmitted to the offspring, whilst the RJF group remained unaffected. These results, taken together, could be interpreted as reduced plasticity due to early life experiences, and effects seem to be transgenerational.

3 Epigenetics

The frequency of unusual phenotypes in the domestic silver foxes after only 20 years of selective breeding were 10^2 to 10^3 times higher than what could be predicted from spontaneous mutation, and the appearance of several new phenotypes together are statistically implausible should they be caused by mutations at structural loci (Belyaev, 1979; Drake, 1999). Selecting for behavior changes the endocrine system, which in turn could affect ontogenic development, which is an effect Belyaev chose to call destabilizing selection. As mentioned above, stress play an important role in its potential to break homeostasis and ontogenic patterns of gene activation/inactivation, and could be an important factor in the accelerated evolution. With genes linked to behavior and hormonal pathways becoming fixed within population in 10 generations, and the aforementioned destabilizing effects that follow (Trut et al., 2009), new routes of genetic research might be of interest when "dissecting" domestication.

The term epigenetics was first used by Conrad Waddington around 70 years ago, and indicate a level of genomic regulation that lies "above" the genetic code, in environmental influence (Waddington, 2012). Waddington (1961, cited by Pigliucci and Murren, 2003) later suggested that if selection acts on acquired characters, what we today call phenotypically plastic traits, they can become inherited characters over the span of time of several generations (Pigliucci and Murren, 2003). A great deal of research in molecular genetics has looked on changes directly to the genome, such as mutations or insertions/deletions (indels), but focus in certain areas has nowadays shifted towards epigenetic changes, and what is rapidly expanding into well characterized epigenomes for different species. Being a buzzword of the 21st century, epigenetics has been covered in numerous articles, and many aspects are under

extensive review. Fields such as pathopsychology and medicine, especially cancer research, have a great interest in mapping epigenetic differences between healthy and sick tissues and cells and creating so called methylomes based on DNA methylation patterns for diagnostic purposes. Others focus at environmental stimulus on the organism of interest and how phenotypic and behavioral changes caused by that are inherited across generations; some of these will be presented and discussed later on in the text.

The transmission of the epigenome is a form of soft inheritance (Richards, 2006); in contrast with what would then be “hard inheritance” of changes such as mutations. The epigenome is more dynamic than the genome, and can be used to fine-tune gene expression in order for an organism to adapt to environmental changes (Bonasio et al., 2010; Jaenisch and Bird, 2003). There has been a shift in how researchers perceive epigenetic marks; DNA methylations have for long been considered stable and less plastic, essentially permanent, compared with histone modifications that are thought of as ephemeral and more easily reversed, maybe due to the fact that there have been gaps in the knowledge surrounding regulatory mechanisms of these epigenetic marks.

The definition of epigenetics has changed slightly with the authors. Waddington (cited by Pigliucci and Murren, 2003) focused on epigenesis, and how phenotypes come about from the genotype during development. Arthur Riggs and his collaborators (cited by Bird, 2007) define epigenetics as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence". The most popular and used definition is that of Adrian Bird (2007) which defines epigenetics as: “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states”. This definition is favored in the sense that it includes many types of regulatory mechanisms, such as DNA methylation and histone modifications.

A big discussion within a certain subfield of epigenetics has been whether somatic cells should be included in the concept or not when discussing the transgenerational transmission of acquired changes. Somatic cells only divide by mitosis, and therefore only transmit genetic and epigenetic memory from cell to cell within a tissue in one single organism, while germline cells that divide through meiosis passes most of the

genetic information the cells have stored on to the next generation. This germline-dependent transmission from one organism to its offspring, are by some authors the only true transgenerational epigenetic inheritance (Crews, 2011). By this definition, context-dependent epigenetics, where generation after generation of an organism is exposed to the same stimuli and thereby is developing the same phenotype are not epigenetically transgenerational since no true inheritance can be determined if the somatic cells are affected continuously throughout generations. The possibility that an effect on somatic cells can somehow convey to the germline should however not be excluded, even when discussing cellular epigenetic inheritance and the bottleneck that transmission only through the germline encompass (cellular transgenerational epigenetic inheritance is extensively reviewed by Jablonka and Raz, 2009).

This in turn brings up an important question surrounding heritable transgenerational effects, that is, at what generations to actually start looking for them? If the F0 generation in for example female rats are exposed to an environmental stimulus or drug during gestation, the offspring, or F1 generation, will in its turn also be affected. However, expanding on this effect of exposure, the germline cells in the F1 generation, namely those that will spawn the F2 generation, will also be directly affected by the physiological response to the environment or drug. Reasonably, it is first in the F3 we can draw any conclusion whereas the effect is truly transgenerational or not. For paternal effects, the F0 and F1 generations will be affected by a stimulus, so transgenerational effects can be determined first from the F2 generation. This is discussed further by Skinner et al. (2008), where he introduces the term "multigenerational" to describe a stimulus that would affect generations F0-F2.

3.1 DNA methylation, an epigenetic mark

One of the most well studied epigenetic modifications is DNA methylation. These nonmutagenic modifications are common among eukaryotes and both plants and animals have been extensively studied in regard of these variations. In this case, nonmutagenic denotes modifications that do not induce a subsequent change of DNA nucleotides by for example DNA repair mechanisms. DNA methylations occur on the base cytosine, regardless of the eukaryotic organism studied, however the context may

vary when looking at animals or plants (Suzuki and Bird, 2008). Focus here lays on animals; in them the addition of a methyl group on cytosine residues in the genome occur in dinucleotides known as CpG. The abbreviation stands for cytosine-phosphate-guanine, simply indicating that a cytosine base is followed by a guanine on the same DNA strand, in contrast with C/G base pairing in the double-stranded DNA molecule. CpG dinucleotides throughout the genome are subject to increased risk of elimination through the process of spontaneous deamination, as have been shown by a lower-than-random occurrence of CpGs in the genome (Krawczak et al., 1998). There are however small areas in the genome that contain a higher-than-normal density of CpGs, where cytosines and guanine have clustered and cover stretches of several hundred bases, forming what is known as CpG islands (CGI) (Cooper et al., 1983; Illingworth and Bird, 2009). These CpG islands can sometime span the promoter regions of genes, and differences in methylation of CpGs provide a regulatory mechanism for gene expression. For example, a study by Eckhardt et al. (2006) on three human chromosomes found a variation in methylation patterns for these CpG islands, with 30% of all CpG islands being devoid of methylation (hypomethylation, less than 20% total theoretical methylation) and slightly more, 40%, having a high degree of methylation (hypermethylation, over 80% of total theoretical methylation).

Promoter region CpG methylations that occur upstream of exons are a well studied and known type of methylation, and they are regarded as repressive marks that downregulate gene expression. Studies have shown that only 20% of tissue-specific genes have CpG-islands in their core promoters, compared to almost 80% of the housekeeping genes (Zhu et al., 2008). The authors suggest that the transcription site GC-boxes in CpG islands allow for a more general mechanism of transcription initiation, something that allow for a basal transcriptome to form from the housekeeping genes.

3.1.1 Global DNA methylation

Global or genome-wide DNA methylation focuses not on local CpGs for a selected gene, but on CpGs across the genome or in repetitive elements when primary candidate genes are not an option. Large scale analysis of promoter CpGs throughout the genome

can give insight in for example pathologically or nutritionally caused methylation differences, and differences can also be combined with gene expression data from array studies. Several methods exist for analyzing DNA methylations both at local and global scale and most of them are based on a technique that chemically alters the DNA based on methylation patterns, namely bisulfite treatment. The idea behind it is that methylated cytosines are protected from the bisulfite and remain intact during the treatment, whilst unmethylated cytosines throughout the genome are converted into tyrosine (Frommer et al., 1992). What remains are small changes in the genome where the methylated cytosines exist, and single CpGs are basically equivalent to single nucleotide polymorphisms (SNPs), and single locus changes can be detected in the same manner as SNP markers. Determining the status of global DNA methylation can be achieved by looking at repetitive DNA sequences or elements, restriction enzyme digestion or pyrosequencing and bisulfite PCR, and focus of such studies in mammals has mainly been on the repetitive elements LINE-1.

A few studies have looked at the prevalence, distribution and classes of transposable elements (TEs) in chicken, and the phylogeny of TEs has been mapped within a number of avian species (Abrusan et al., 2008; Kaiser et al., 2007; Wicker et al., 2005). Wicker et al. (2005) sequenced and characterized high-copy DNA, or TEs, and found several novel repeats and also subfamilies of already known classes such as CR1, and CNM, a 40bp short tandem repeat. Approximately 10-15% of the chicken genome consists of TEs, compared with the 45-50% in humans. Around 80% of the repeats are made up by CR1, and approximately 200 000 copies of CR1 have been annotated (Int Chicken Genome Sequencing, 2005). CR1 are characterized by a CG-rich internal promoter just like the mammalian counterpart LINE-1, and has been extensively investigated for length, chromosomal distribution and correlations with CG-density in the genome (Abrusan et al., 2008; Liu et al., 2009).

3.1.2 The regulation of DNA methylation

3.1.2.1 DNA methyltransferases

The exact processes of regulation and maintenance of DNA methylations are still not fully understood. Today, we know that three types of DNA methyltransferases (DNMTs) are responsible for catalyzing DNA methylation in mammals, and they are distinguished into two types. DNMT1 in mammals has been recognized as the enzyme responsible for upholding methylation patterns, as it is a part of the replication machinery and act on the hemimethylated DNA that DNA synthesis generates (Bostick et al., 2007; Robert et al., 2003). As CpGs create palindromes on the complementary strand, methylation patterns can be identified and faithfully replicated on the new strands. Another feature of DNMT1 is its ability to bind to unmethylated DNA, which will block the unmethylated CpGs and thereby prevent *de novo* methylation (Song et al., 2011).

The two other methyltransferases are named DNMT3a and DNMT3b and their roles include the regulation of methylation throughout development and to some extent also dynamically alter methylation patterns as a response to environmental stimuli (Okano et al., 1999). In Apis larvae for example, diet changes to the methyl donor rich royal jelly promotes queen development (Kucharski et al., 2008). Silencing of Dnmt3 in larvae can induce queen-like individuals with fully developed ovaries to form, by changing what epigenetic memory will form in these developing organisms. Other examples of environmental stimuli affecting DNMT activity will be presented further on in the text.

As for methylation models and mitotic inheritance, a study on cancer cells has categorized 5 distinct patterns: high, low and random methylation, and stochastic high or low (Teng et al., 2012). Plain high or low patterns signify that they are kept at the same methylation levels generation after generation, whilst a stochastic high pattern is one that will show a gradual increase in methylation after a few generations, and vice versa for a stochastic low pattern. A highly enriched transcription binding site number was found for all non-random loci, which in turn suggest a cis-regulatory effect on these locations and genes in order to keep up methylation fidelity. The results lead the authors to suggest that the effectiveness of both methylation maintenance and de novo methylation affect the stability of epigenome patterns throughout cell divisions.

Instability of DNA methylation marks or spontaneous *de novo* methylation could be reason for an increasing epigenome variation over several generations, and might not be as uncommon (Schmitz et al., 2011).

3.1.2.2 Demethylation

Whilst error rates for single-base substitutions during mitosis vary but are in the ranges from 10^{-3} to 10^{-6} depending on factors like the polymerase or sequence, the maintenance efficiency for DNA methylation at hypermethylated CpG islands is at ~96%, meaning one out every 25^{th} methylated cytosine is lost (Kunkel and Bebenek, 2000; Laird et al., 2004). The disadvantage of C to T mutation occurring at methylated cytosines is a risk apparently compensated for by the ability to roughly regulate expression.

As for active demethylation the questions are still abundant, the hunt for global demethylases have not yielded any breakthroughs as of yet. Some demethylases do exist, like the methylated DNA-binding protein MBD2 (Bhattacharya et al., 1999). The occurrence of MBD2 in the testis as well as somatic cell could possibly allow plasticity even in differentiated and fully developed cells. A long ongoing investigation has been in regard of how or if epigenetic marks persist through gametogenesis and fertilization. It is known that epigenetic marks are used for imprinting and X chromosome inactivation (Li et al., 1993; Panning and Jaenisch, 1996), and the general consensus up until recently has been that the embryos undergo a big reprogramming event, with global demethylation as one of the driving factors, and that these marks would be remembered in an yet unknown way and reestablished after a few cell divisions (Dean et al., 2001; Santos et al., 2002). Some studies have reported contradictory results in other organisms than rats and mice, and suggest that the mammalian epigenetic reprogramming event is not a requisite for all species (Beaujean et al., 2004; Shi et al., 2004). Recently, a study by Li and O'Neill (2012) neatly challenged the paradigm and whole concept of global active demethylation during fertilization, and showed that the reported demethylation in previous studies has been due to a masking of the antigens used in immunolocalization. The analysis of methylated CpG levels in fertilized mouse zygotes during the first few cell-cycles concluded that methylation levels are indeed preserved even through fertilization. The authors go on to speculate that the underlying

cause of the antigen masking might be due to either chromatin structure or a conformation change.

One potential pathway for demethylation is through hydroxymethylcytosine (5hmC), a modified variant of cytosine similar to methylated cytosine. The role of 5hmC is not fully elucidated, but it has been suggested to be involved in the regulation of DNA methylation (Bhutani et al., 2011). TET proteins (ten-eleven translocation proteins) are involved in the hydroxylation of 5mC into 5hmC which can then via passive or active DNA demethylation result in cytosine replacement. TET proteins are suggested to regulate DNA methylation integrity, and TET inactivation could be one factor responsible for the DNA hypermethylation seen in cancer (Williams et al., 2012). Another pathway for demethylation is the deamination of methylated cytosine into thymine by activation-induced cytidine deaminase (AID) (Guo et al., 2011). The thymine is then replaced by cytosine via mismatch recognition and base-pair repair, and AID has even been suggested to work together with TET.

3.2 Histones and histone modifications

The chromatin, and within that the nucleosome complex, is the wrapping of DNA around a histone octamer. This permits a tight packing of DNA within the nucleus. The histone octamer consists of the core histones H2A, H2B, H3 and H4, all in duplicates. Histone tails can be subject to post-transcriptional modifications (PTMs), changing the chromatin structure and altering it between the transcriptional permissive euchromatin, and the condensed heterochromatin (Jenuwein and Allis, 2001). Before going on to the details of PTMs, it is worth mentioning that the histones H2A and H3 come in different variants, all highly conserved within each group but still with distinct different purposes. Human H2A have two important variants; H2AX, which seem to mark DNA double-strand breaks, and H2AZ, present in transcriptionally active sites (Redon et al., 2002). Human H3 come in five variants, H3.1, H3.2, H3.3, centromeric protein A (CENP-A, found specifically at the centromeres), and H3t, found in the testis (Loyola and Almouzni, 2007). Just as CENP-A is linked to a specific region on the chromosome, a suggestion is that the three non-centromeric variants H3.1-H3.3 are located and mark different types of chromatin. The idea behind this is that the different

histone variants would contain different PTMs that could aid in their remodeling of the chromatin. H3.1 contains both transcriptionally repressive and activating PTMs, whereas H3.2 is mainly marked with repressive PTMs different from those in H3.1, and H3.3 contains PTMs connected to transcriptionally active euchromatin (Hake et al., 2006). The diversity of histone variant levels could be tissue-specific, or ontogenic. H3.3 can be employed independent of replication and aggregate at active loci, whilst the deposition of H3.2 is replication dependent (Ahmad and Henikoff, 2002). H3.1 is incorporated both during and outside the cell cycle S phase, and has also been shown to mark DNA repair after UV damage, where newly synthesized H3.1 histones are used (Polo et al., 2006).

3.2.1 Maintenance after replication

An important topic regarding the histone code is the question of how it remains after chromatin replication. Current theories suggest that parts of old histones tetramers are integrated with newly synthesized core histones in a random fashion, and restoration of PTMs continues in the daughter cells (reviewed by Alabert and Groth, 2012; and Probst et al., 2009). With repressive lysine (K) methylation as an example, the monomethylation restoration seems more rapid than the trimethylation, but even with a slight delay in restoration no notable change of transcriptional activity has been noted for newly synthesized strands, which suggest that the histone PTMs that do survive replication are enough to reach the silencing threshold (Alabert and Groth, 2012). In the germline, sperm cell histones are exchanged for protamines to ensure an even more tight packing of the genome, and they are rapidly replaced by histones immediately after fertilization (Morgan et al., 2005; van der Heijden et al., 2005). An interesting finding is that the newly replaced histones are of variant H3.3 for the paternal chromosome (van der Heijden et al., 2005), and this could be a way for the cells to distinguish between maternal and paternal chromosomes and in turn facilitate imprinting.

Histones are in a dynamic relationship with DNA, and it has been shown that nucleosome positioning will affect the patterns of DNA methylation around it (Chodavarapu et al., 2010). Others have studied nucleosome formation looking at it from the other direction, concluding that preexisting DNA methylation patterns will

influence nucleosome assembly (Perez et al., 2012). Histone modifications, or PTMs, have as mentioned an important role in regulation of transcription, with the two by far most important modifications being acetylation and methylation (for reviews, see Jenuwein and Allis, 2001; Kouzarides, 2007; Strahl and Allis, 2000). This histone code has the potential to change interactions with the chromatin and by that alter downstream cascades, and as mentioned before, the location of the modifications is of great importance. Information of the two major PTMs and their effect on transcription are given below, and examples about correlation with transcriptional status and DNA methylation patterns in relation to behavior is found in paragraph *Behavioral epigenetics*.

3.2.2 Histone Acetylation, the activator

Starting off with acetylations, it is a modification generally associated with active transcription, and deacetylation is associated with transcriptional repression. It alters chromatin structure, relaxing it, by neutralizing the amino group in lysine residues and its positive charge (Brownell and Allis, 1996). This will result in a decreased attraction between the DNA and histone tail, which leads to formation of euchromatin. Gene-rich DNA regions show the highest abundance of H3 acetylation, keeping the chromatin in a relaxed state, and these regions are also hypomethylated which could be due to acetylations preventing the recruitment of DNMTs (Roh et al., 2005). Roh and collaborators found that diacetylation (K9/K14) of H3 is correlated with CpG islands, but not uniformly with all active genes in the genome. Besides K9 and K14, acetylations can occur at several amino acids of histone tails but few have been found; K18, K23 and K27 for H3, and K5, K8, K12 and K16 for H4, with 11 theoretical acetylation sites remaining (He and Lehming, 2003).

3.2.3 Histone Methylation

Methylations have no effect on the electrostatic properties, so the histones inherent positive charge reduces transcription by interacting strongly to the negative DNA (He and Lehming, 2003). The role of methylations seems to be more linked to signal for

recruitment, or as mentioned earlier, altering downstream cascades (examples in Yun et al., 2011). The modification is primarily found at the residues lysine and arginine, with monomethylations linked to active transcription sites and trimethylation found at sites with repressed transcription (Barski et al., 2007). Scans of H3 have found the lysines 4, 9, 14, 27, 36, 64 and 79, as well as arginines 2, 17 and 26 all can be methylated (Loyola and Almouzni, 2007; Zhang et al., 2002). The transcriptional repression by H3K9me (methylation of histone 3, amino residue 9) is achieved by the affinity of heterochromatin-associated proteins to bind to that modified residue (Rice and Allis, 2001). Methylation of H3K9 is only achieved after the removal of acetyl groups on that same residue, and methylation of K9 has been shown to be required for DNA methylation (Tamaru and Selker, 2001).

One residue of special importance is H3K4, a marker for active gene expression, and the different states of methylation that can occur on it. An interesting finding is that dimethylation, H3K4me2, is found in all CpG island promoters as if it is an innate feature of CpG islands (Weber et al., 2007). DNA methylation seems to be dependent on the CpG content in promoters, resulting in constitutive hypo- or hypermethylation, and H3K4me2 enrichment prevents DNA methylation in inactive promoters. H3K4me2 occurs at both active and inactive genes, whereas the tri-methylation of H3K4 is a marker for active transcription (Santos-Rosa et al., 2002). One study looked at predicted enhancers and found that most were conserved, and marked by cell-specific chromatin modifications (Heintzman et al., 2009). Promoters, enhancers, insulators and cis-regulatory elements drive cell-type-specific gene expression, and H3K27ac were enriched in enhancers in general, whilst H3K4me1 was occurring in a more cell-type-specific fashion.

4 Epialleles: Agouti and Axin fused

Alleles differ from each other by nucleotide mutations. Epigenetic alleles, or epialleles, differ by having different DNA methylation patterns. Regarding epialleles, there exist two widely known cases in mammals, both in mice; in the agouti gene and the axin fused. The agouti genes encodes a signaling protein that gives the mice its wild-type

color by switching the synthesis of eumelanin to pheomelanin, which causes a yellow sub-apical band to appear the otherwise black hair. Several dominant mutations of this allele exists, the most studied being A^{vy} (viable yellow), a mutation that carry an intra-cisternal A particle (IAP) containing a cryptic promoter, upstream of the agouti gene (Morgan et al., 1999). The promoter changes the expression pattern depending on its epigenetic status, namely promoter CpG methylation, and the methylation patterns are heritable even through meiosis. By studying isogenic mice researchers have found that the hypermethylation silences the gene while correlating with H4K20me3 enrichment and hypomethylation is linked to histone acetylation and gene expression (Dolinoy et al., 2010). The epigenetic patterns of the gene are unstable, which give rise to phenomena such as variegation between cells and variable expression between individual, and by that different phenotypes ranging from agouti to pseudo-agouti (Rakyan et al., 2002). Since the epigenetic patterns can be influenced by factors such as nutrition the transmission of the alleles do not follow a strict mendelian pattern of inheritance, and alleles such as this have been suggested to be referred to as metastable epialleles (Rakyan et al., 2002; Waterland and Jirtle, 2003). Axin fused ($Axin^{Fu}$) is a gene implicated to regulate embryonic axis formation (Vasicek et al., 1997), and much similarly to agouti the mutations of the gene are caused by the insertion of an IAP in the sixth intron (*Fused*). *Fused* produces a kink-tailed phenotype, or not, depending on the methylation status of the IAP insert. Except for the IAP insertion in both these alleles, what the mutations have in common is that they also display pleiotropic effects in addition to those mentioned above. Agouti has been studied in respect to the adult-onset obesity that A^{vy} cause, along with a small but significantly increased risk of tumor development (Duhl et al., 1994). Except for the tail kinks, axin fused has been linked to the occurrence of deafness and neuronal effects (Zeng et al., 1997). Understanding of heritance patterns and susceptibility of epialleles to external or internal stimuli might give us better insight in imprinting processes and activation of proto-oncogenes.

5 Behavioral epigenetics

As of today, reliable studies on transgenerational behavioral epigenetics (i.e. germline-dependent) are scarce, if not even nonexistent. In a report from a meeting on behavioral

epigenetics a few years ago, Lester et al. (2011) reported that a literature search at that time for citations on the subject of behavioral epigenetics only generated 96 articles. That list is not fully complete, but is an indication on how small this research area is at the moment. From the list, the authors arranged the publications into different clusters based on construct, and in each construct grouped them by the genes studied in those publications. The big main constructs came to be substance use, parenting, learning/memory, psychiatric illness and stress. Psychiatric illnesses are not discussed here per se, but examples from the rest of the constructs will be reviewed in the following pages. The effects of epigenetic variation on factors like susceptibility to factors like psychiatric illnesses should however not be dismissed. An example is catechol-O-methyltransferase (COMT) activity. COMT is an enzyme involved in inactivation of DA in the prefrontal cortex (PFC), and promoter region hypomethylation has been correlated with schizophrenia and bipolar disorder (Abdolmaleky et al., 2006). In COMT exon 4, there exists a non-synonymous SNP exchanging G to A, Val to Met that eliminates a CpG site, which also lowers COMT activity and increases stress sensitivity (Ursini et al., 2011). Data suggests that conserved Val is unmethylated during stressful situations to keep up activity of expression and protect against stress through inefficient PFC activity, something lost in the mutation.

5.1 The influence of drugs

Starting off with drugs, the examples given here are based on the effects of alcohol and chemical agents. Bonsch et al. (2006) looked at longtime exposure of alcohol in human alcoholics and found a 10% total DNA methylation increase. These changes show a negative correlation with the levels of DNMT3a and 3b, and the decrease of DNMT3b was also negatively correlated with high blood alcohol levels. The authors speculate that alcohol levels were not a direct cause for the DNMT changes but instead act through some feedback loop, thereby disrupting methylation regulation. Using Agouti mice, alcohol exposure *in utero* has been shown to generate a variety of developmental effects, such as differences in methylation patterns and body weight, impaired growth, and site-specific transcriptional silencing (Kaminen-Ahola et al., 2010). The epigenetically sensitive A^{vy} allele in Agouti mice can be used as an indicator for

epigenetic changes. In this study, not only was this gene silenced, a gene expression array confirmed significant up- and downregulations of other ones as well, but no global methylation differences were found when looking at genome-wide IAP methylation.

The epigenetic effects of prescription drugs are under investigation as potential unstable side-effects might influence behavior in unintended ways. Morphine exposure in adolescent rats leads to a decrease in both nursing and pup grooming frequency, and higher off nest frequency (Johnson et al., 2011). This affects the pups, altering their play behavior and making male offspring being less involved in rough and tumble play, and females engage more in such activities. In another study, paternal cocaine use led to sex-linked effect in the offspring, making male progeny more resistant to cocaine addiction (Vassoler et al., 2013). The resistance correlated with increased PFC brain-derived neurotrophic factor (*Bdnf*) expression and H3ac of *Bdnf* promoters. Toxic exposure can also lead to behavioral changes, as shown in a study on rats treated with the fungicide vinclozolin (Skinner et al., 2008). Vinclozolin has been shown to work as an endocrine disruptor and induced large, sex-specific alterations in gene expression in hippocampus and amygdale, with minimal overlap between genes in the two areas. The altered transcriptomes in males and females led to distinct differences in behaviors, such as anxiety, with males showing a reduction in anxiety-like behavior. The expression and behavior changes are transgenerational and effects of a single exposure can be observed even in F3 generation.

5.2 Nutritional change and challenge

Mentioned in an example earlier, nutritional supplements can affect bee larvae development (Kucharski et al., 2008). Several other studies have looked at similar changes in both bees and ants, trying to map both the transcriptome and developmental changes that the caste separation induces (Foret et al., 2009; Lucas and Sokolowski, 2009).

An interesting research topic is the transgenerational effects of nutritional change, or challenge, and one such study is that of the Dutch famine and its consequences on developmental programming. Recent studies have claimed to be the first to provide

evidence in humans that epigenetic changes caused by transient early-environmental conditions are constant through life, with reports of elevated risk of cardiovascular related diseases, metabolic changes and alterations of blood lipid levels (Heijmans et al., 2008; Tobi et al., 2009). The condition in this case was nutritional deprivation due to food rationing in the western part of the Netherlands during the winter of 1944, and the studies have focused on the effects of prenatal exposure on the now adult individuals. Researchers have found changes not only depending on the state of pregnancy, but also sex-specific effects (Tobi et al., 2009). Periconceptional exposure to famine induced sex-specific metabolic differences and different methylation patterns for selected loci, whilst starvation during late gestation caused no noticeable changes. Among the findings are changes in hyper- and hypomethylation for several loci, most notably the IGF2 gene, an insulin-like growth factor responsible for development and growth (Smith et al., 2006). Most recently, the same research group also concluded that focusing in continued studies must be on selected genomic regions, after reporting no significant difference in global methylation between people with prenatal exposure to famine and unexposed control, using LINE-1 LTRs as reference (Lumey et al., 2012).

Other authors have reported similar regulatory changes using nutritional change (Burdge et al., 2011). In rats, a 25% nutritional increase was introduced in the F0 generation during pregnancy and kept at this level until the F3 generation. The nutritional alteration caused changes in regulation of glucose and fatty acid metabolism in the offspring, with fasting plasma glucose levels increasing over the span of several generations. A decrease in the methylation pattern of *Dnmt3a2* promoters was found between the F1 and F3 generations, correlating with changed expression patterns to a more active state. These context-dependent changes are a good example of the adaptive capability to environmental stimulus such as nutrition.

5.3 Learning and stress

5.3.1 Glucocorticoid receptors and maternal care

In research regarding learning and stress the obvious tissues to study is the hippocampus and the amygdala, as they are involved in memory formation and mediates emotions as

for instance anxiety, and maternal effects have shown to shape the epigenetic memory and influence the behavior of the offspring. In addition to these, the hypothalamus is a central part of the stress response pathway, and changes therein will propagate throughout the HPA-axis as mentioned in the *Stress* paragraph.

Licking and grooming (LG), a trait extensively studied in rats, is passed on from mothers to their offspring. Other than the display of that trait itself, it influences the epigenome to the degree that it alters physiological responses to stress. These changes can come about due to the causal relation between histone modifications, DNA methylation, glucocorticoid receptor (GR) expression, and binding of NGF-inducible protein A (NGIF-A) to the GR promoter (Weaver et al., 2004). In addition to these changes, an altered HPA-axis response to stress was also shown. To begin with DNA methylation, all rat offspring of the strain used in the experiments are born with a methylated exon 1_7 GR promoter (referenced here on only as GR or GR promoter), no matter what behavioral LG pattern (high/low) the mothers are expressing (Weaver et al., 2007). During the first week after birth, rearing by high LG mothers will cause a demethylation in the exon 1_7 GR promoter in the pups (Weaver et al., 2004). Methylation patterns are reversed with cross-fostering, indicating that the biological mother is not the contributing factor to the methylation status. In addition to changes in DNA methylation, the active chromatin mark H3K9ac was studied and found to be significantly more abundant in high LG reared offspring, and this was associated with a great increase in NGIF-A protein binding to the GR promoter.

The association between DNA methylation status and histone modifications has been further investigated by the means of both nutritional supplements and toxins. L-methionine supplement acts a methyl donor and reversed GR demethylation in high LG mother, inducing downregulation of GR along with a large number of other genes, just as expected from a higher degree of DNA methylation (Weaver et al., 2005). The GR expression reduction correlated with increased corticosterone levels as a response to restraint, and also altered behavior in the forced swim-test, matching that of low LG mother raised pups. The organic compound Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, increased H3K9ac and with that also binding of NGIF-A to the GR promoter in low LG adult offspring (Weaver et al., 2004). It also caused demethylation

of the GR promoter, along with upregulation a small subset of genes (Weaver et al., 2006). When bound to the GR promoter, NGFI-A induces promoter activity, and NGFI-A over-expression induces promoter demethylation by residual binding to the promoter. When examining cell cultures from hippocampus, addition of serotonin stimulated NGFI-A dependent DNA demethylation, comparable to *in vivo* high maternal care effects (Weaver et al., 2007). Estrogen receptor-alfa b (ER-αb) methylation has also been examined in rats, and low LG mothers increased promoter methylation and decreased expression (Champagne et al., 2006). The elevated methylation correlated with lower binding of transcription factor Stat5b to the ER-αb promoter. Comparable with result from studies of the GR, the expression and methylation effects were rescued by cross-fostering.

In humans, maternal mood (expressed as anxiety or depression) during the second and third trimester increased methylation of the NGIF-A consensus binding site in exon 1F (human homologue to rat exon 1_7) in the human GR gene *NR3C1* in blood samples (Oberlander, 2009). Different CpG sites in the exon displayed varying methylation during different trimesters, indicating different promoters being important during different developmental periods. Global methylation (LINE-1) was however not affected by mood changes. The effects also correlated with elevated infant cortisol response levels, indicating an effect on the HPA-axis.

As conclusion, mother-infant interaction form the offspring for life, but the changes are not set in stone as they show signs of dynamic alteration. These findings indicate that certain early-life experiences can be rescued or altered even in adulthood.

5.3.2 Fear and memory

In regards of learning and stress, another gene of interest is *Bdnf*, which is vital for synaptic plasticity and maintenance of long-term memory, something studied in relation to fear and stress. As mentioned above there is plasticity in neural development, and early-life experiences such as adversity form individuals. A study on rats showed that pups raised by stressed mothers had increased PFC *Bdnf* methylation, effectively lowering gene expression (Roth et al., 2009). The effects of the maltreatment carried on to the next generation (F1), and cross-fostering with caring parents did not rescue DNA methylation patterns. In contrast with aforementioned context-dependent alterations in

GR in LG mice, these effects seem transgenerational in a genetically transmitted manner. It seems like there exist a window of time for when pups are susceptible to the effects of stress, as adult rats exposed to stress do not show any persistent epigenetic changes (Fuchikami et al., 2009; Lubin et al., 2008).

Different exons in *Bdnf* are regulated independently depending on the type of fear conditioning or related stimuli, indicating different mechanisms for different memories (Lubin et al., 2008; Miller and Sweatt, 2007). In the study by Lubin et al. (2008), chromatin remodeling was investigated as factor in memory formation and the upregulation of *Bdnf* expression caused by fear conditioning. The expression effects dropped back to baseline within 24h. The same result were seen when animals were treated with an DNMT inhibitor, which caused demethylation along with increased H3ac and H3 phosphoacetylation, correlating with upregulation of different exons. An H4ac decrease was found but was not correlated to expression differences, which could imply a hierarchy among modifications and exons.

Acute stress by immobilization in rats leads to a decrease in hippocampal *Bdnf* expression and protein levels, results correlated with a decrease of acetylated H3 in three of the four *Bdnf* promoter regions (Fuchikami et al., 2009). None of these effects were found 24h after immobilization, indicating plasticity in H3 acetylation and a quick way of regulating expression by chromatin remodeling. Acute stress also influences H3 methylation levels, with an increase in H3K9me3 levels and a decrease in H3K9me1 and H3K27me3, the latter mark connected to transcriptional repression (Hunter et al., 2009). Treatment with corticosteroid stress hormones did not induce the same changes suggesting that the histone variations and effects of stress are transmitted through other neurotransmitter pathways, possibly by catecholamines or glutamate. Drug-induced fear extinction has shown to cause histone modifications around the *Bdnf* gene promoters but only in prefrontal cortex, not in amygdala or hippocampus (Bredy et al., 2007). Mainly, H4ac increased with the extinction of conditioned fear, implying a mechanism for regulation of long-term memory. These acetylations showed an inverse correlation compared to acetylations of H3, implicating that different histone codes could be used for various types of learning and memory formation.

Fear conditioning in rats upregulate hippocampal *Dnmt3a* and *3b* gene expression and also methylates protein phosphatase 1 (*Pp1*), a memory suppressor gene (Miller and Sweatt, 2007). Inhibition of DNMTs abolishes *PP1* methylation and thereby disrupts proper memory formation. The gene *Reelin*, involved in enhancing long-term potentiation (LTP) and memory formation, was demethylated by fear conditioning, and even further demethylated by DNMT inhibition. All effects of DNMT inhibition were gone after 24h, indicating a reversible and dynamic regulation which suggests that DNMTs is involved in short-term but not long-term memory formation.

Inhibition of HDAC in mouse hippocampus enhances memory for contextual fear conditioning, but not cued (Vecsey et al., 2007). Fear conditioning seems to be mediated via the transcription factor cAMP response element-binding protein (CREB) and coactivator CREB-binding protein (CREB:CBP) pathway, which promote LTP when acetylations are abundant. Gene analysis showed an upregulation of *Nr4a2* (same receptor subfamily as *Nr4a1*, aka *NGFI-B*) after fear conditioning and HDAC inhibition, indicating its importance in this pathway of memory consolidation.

In a final example of epigenetic regulation, repressed transcription by heterochromatin formation can be caused through transcription factor KRAB and its coactivator KAP1 (Jakobsson et al., 2008). Hippocampus KAP1-knockout mice were more sensitive to stress during learning tasks, and also showed heightened anxiety and exploratory behavior. These results correlated with higher levels of activation markers H3ac and H4ac and a decrease of silencing H3K9me3, and the results demonstrate a pathway for modulating stress and how epigenetic regulatory mechanisms are modified through it.

6 Concluding remarks

DNA methylation and histone modifications, epigenetic marks that alter the chromatin structure and by that act like regulatory mechanism, are key factors in new research fields such as behavioral epigenetics. Their ability to not only shape individuals but also their progeny makes them hot targets when investigating the effects of environmental conditions or stimuli on an organism. As briefly shown in this essay, there are several

examples of situations where memory and associations that enable us to learn are modulated or even disrupted by epigenetic marks, as with condition of fear. Variation of nutrients or amounts of it forces organisms to adapt to the changes as much as they can, and linking back to Waddington's initial idea of epigenesis, inherited or context-dependent changes form new individuals and shape them according to an expected milieu. These changes can be unfavorable for an organism if the expected milieu suddenly where to changes again, and the dynamic properties of epigenetic regulation are not enough to shift back, as seen in the Dutch famine study. The short intervals of susceptibility to change, and their long-lasting effects, are what make epigenetic regulatory mechanisms such an important research field. Knowledge about what triggers epigenetic variation, and in some cases how to counteract them can be applied within areas of our interest, as for instance the effects of stress and housing on animal welfare in fowl. Further insight in processes such as domestication might also shed light on the nature of selection upon epigenetic differences and their propagation.

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