Epigenetic changes in the developing brain: Effects on behavior

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This Sackler Colloquium encompasses a broad range of topics for the following reason. Our knowledge of the chemistry of epigenetic modifications is expanding at a rapid rate, but most of the primary discoveries in this field are made using nonneural tissue. So, neuroscientists want to learn about this chemistry but may not have direct exposure to the material. In a complementary fashion, molecular geneticists and protein chemists who experiment on DNA methylation, histone modifications, and noncoding RNAs realize that some of the most exciting applications of their discoveries are in the CNS, but for such scientists behavioral assays, for example, are distant from their expertise. The purpose of this Sackler Colloquium, therefore, was to bring together experts in the two fields—epigenetic chemistry and behavioral neuroscience—in the spirit of mutual education.

We start with the chemistry of transcription itself. Decades of biochemical work led to the concept of the basal transcriptional complex (1, 2), and recently a structure for the transcription preinitiation complex was reported (3). Neuroscientists concerned with the development of behavior need to know the variety of changes in the cell nucleus that precede the formation of that complex, with an emphasis on those modifications that last a long time.

It has been established that specific genes operating in specific neurons govern the appearance of specific, biologically crucial behaviors. The system that has been worked out in the most detail features estrogen-dependent female reproductive behavior (4, 5) modulated by estrogen-facilitated transcription of a range of genes in ventromedial hypothalamic neurons required to activate a spinal-midbrain-spinal circuit. Similarly, expression of the gene encoding the estrogen receptor-α (ER-α) in medial preoptic neurons is essential for the performance of maternal behaviors (6). For behavior and other physiological functions, early papers reported that the first molecular event that leads to hormone-facilitated transcription, depends on the binding of ER-α to an estrogen response element (7), followed by the actions of coactivator proteins (8, 9). Recent molecular endocrine papers are more ambiguous on the exact order of events (see *Some Outstanding Questions*, below) (10–12). The long-lasting epigenetic changes in the nuclei of hypothalamic neurons, which are responsible for the normal development of this chain of estrogen-dependent reproductive behaviors, have remained obscure.

Thus, the following series of papers in this Sackler Colloquium focus on mechanisms that include DNA methylation, genomic imprinting, histone modifications, and noncoding RNAs, applying this new knowledge to neuronal mechanisms for behavior, wherever possible. Here we offer substantive backgrounds on several types of mechanisms in play.

To date, the behavioral systems most easily explored with respect to epigenetic mechanisms have been those that do not need to be learned. Thus, at the Sackler Colloquium mechanisms discussed were those for maternal behaviors (13), sexual behaviors and stress (14), and hypothalamically controlled behaviors (15). Circadian-regulated behaviors (16) clearly represent a case of normal behaviors epigenetically regulated, whereas problematic and abnormal behaviors, such as in Prader-Willi syndrome (17), and changes in behavior during aging were also discussed. Potential contributions of transposon expression to expanded opportunities for neuroplasticity were discussed at the meeting by Gage, and are represented here among the Outstanding Questions emanating from the meeting.

Epigenetics

In 1946 Waddington (18) introduced the term "epigenetics" to link the phenotype with genotype during development. The notion of heritability became linked to "memory" marks (DNA methylation) for propagating cell identity by structural regulation of chromatin for gene-expression states (register, signal, or perpetuate activity states) (19).

Indeed, epigenetics epitomizes the development of the brain more than that of any other structure. The billions of neurons exponentially magnified for function by the trillions of synaptic interconnections mean that no two brains are alike. Even monozygotic (MZ) twins show differences in behavior and in psychiatric disorders that become more marked with age (20). There are, as reported in this Sackler Colloquium, reports of experience driven heritable changes in the brain's epigenome, especially experiences involving maternal care (13) and stress (21). Neural systems are designed to respond to the environment because the strengths of their synaptic connections are activity-dependent. In humans the accumulation of brain knowledge across generations has played an integral role in shaping and ameliorating environments to optimize longevity and reproductive success.

It is, however, important to distinguish between transgenerational hereditary and intergenerational effects. Examples of the latter include in utero exposure to nutritional status, stress, or toxic environmental factors that act on the developing embryo and its germ line (22). These intergenerational events should be distinguished from truly transgenerational inheritance, which is found in subsequent generations that were not exposed to the initial environmental events that triggered the change.

When considering intergenerational effects it is also important to consider which parent was exposed. Any harmful events experienced in utero are likely to affect the next generation of both males and females. These events can also affect the third generation of the female offspring, as the female germ line develops in utero, where the primordial germ cells undergo demethylation and remethylation. These epigenetic marks are likely to

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persist in the egg, unlike those of the sperm, which are erased after fertilization. In the absence of in utero stressors or toxins, the demethylation/remethylation reprogramming provides a major barrier to transgenerational epigenetic inheritance.

In the context of intergenerational inheritance, the placenta and brain are of particular importance. The fetal placenta is integral to the successful development of the next generation, interacting and regulating much of maternal physiology and behavior, thereby ensuring its own successful development and, in turn, that of its fetus continue. Maternal care interactions continue after birth, further ensuring this next generation receives adequate nutrition, warmth, and tactile stimulation.

DNA Methylation and Demethylation. In development, DNA methylation imposes a fundamental epigenetic barrier that guides and restricts developmental differentiation, represses transposable elements that threaten genome stability, coordinates expression of imprinted genes according to parent of origin, and plays a role in sex chromosome dosage compensation. The evolution and inheritance of such epigenetic mechanisms has shaped gene expression to provide a multitude of cell and tissue phenotypes from a single fertilized egg. To reset the epigenome for totipotency, primordial germ cells are required to undergo sequential epigenetic changes and genomewide demethylation (23). Resetting of maternal DNA methylation barriers requires germ-line reprogramming by tet methylcytosine dioxygenase 1 (Tet1) and tet methylcytosine dioxygenase 2 (Tet2) between embryonic days 10.5 and 11.5 in the female mouse, whereas paternal germ-line reprogramming takes place initially postfertilization in the zygote, where methylation marks are removed by tet methylcytosine dioxygenase 3 (Tet3) hyroxylase and subsequently via passive demethylation via base excision repair mechanisms (24). The base excision repair pathway both protects against cumulative genetic damage and serves as an auxiliary active demethylation mechanism. A restricted number of loci escape demethylation, notably those associated with intracisternal A particle elements, which may be a contributory factor in their escape from reprogramming (25). It is therefore a possibility that rare, but functionally relevant 5mC alleles could be inherited over multiple generations by evading erasure during zygotic and primary germ-cell reprogramming. Finally, we note that in this rapidly expanding field, a wide variety of DNA methylation mechanisms have been reported (reviewed in ref. 26). For example, 5-hydroxymethylcytosine has been reported in Purkinje neurons (27).

Genomic Imprinting. During germ-cell development, genomic imprints are also established, allowing parent-of-origin specific expression of particular genes. The primary origin and the role for the matriline in genomic imprinting (maternal methylation, maternal reprogramming) probably owes much to its evolutionary significance and success in mammalian in utero development. Its contribution to placentation, metabolism, thermogenesis (body temperature regulation), maternal care, growth, and brain development are global functions targeted by imprinted gene regulation.

Genomic imprinting is regulated by epigenetic marks in the imprint control region (ICR) of genes. These marks are heritable and result in the monoallelic, parent of origin-dependent expression of genes. The ICRs are primarily maternally methylated and are inherited and reprogrammed through the matriline (28), with three main exceptions (H19, Rtl1, and Dlk1). Imprinting of retrotransposon-Like 1 (*Rtl1*) and delta-Like 1 homolog (*Dlk1*) genomic regions is thought to have occurred after the marsupial-eutherian divergence, with the accumulation of miRNAs and SnRNAs on the maternal chromosome, which has an active role in the regulatory expression of these genes (29).

The ICRs have gained regulatory control over clusters of genes increasing in number from marsupials to rodents to humans, and of particular importance for tight regulation over gene dosage. The significance of this regulation is illustrated through the complexity of disrupted phenotypes that occur with perturbations to the ICR without structural mutations in the genes themselves (examples seen in Angelman's syndrome, Prader-Willi syndrome, and Silver Russell syndrome) (30). It is important to emphasize this is not a single gene effect and many imprinted genes are coregulated as part of an imprinting network that serves a similar global function (growth, metabolism, maternal care) (31). In addition, each imprinted gene network may itself regulate a network of downstream genes engaged in specific cellular functions (32) (development of pluripotent cells that become committed to produce and respond to signals as part of a global tissue function).

For example, the paternally expressed 3 (Peg3) transcriptome has a network of 22 genes concerned with neural development, and 21 genes that are regulating other transcription factors. Peg3 also regulates a number of genes concerned with placental development (pregnancy specific glycoproteins, ceacams, and prolactins). These genes have undergone multiple duplications across mammalian species (*Psgs 1–23*, *Ceas 1–19*, *Prls 3–18*) with the lowest copy number in metatherians and highest in eutherian primates. An important feature of genes that are imprinted is their evolutionary stability through purifying selection, and the recruitment of more genes to this epigenetic mode of regulation (ICRs) has occurred across the evolution of mammalian species (33). This recruitment has often expanded the ICR to include noncoding RNAs. Imprinted gene clusters contain at least one long noncoding RNA (lncRNA), and two clusters in particular (*Kcnq1-Kcnq1 ot1*; *Igf2r-Airn*) have an estimated 100 kb of lncRNA transcribed in an antisense direction from within the protein-coding genes (34). The lncRNA plays an essential role in the imprinting of these clusters and deletion of their promoter results in biallelic expression.

The imprinted genes are themselves remarkably stable and have not undergone subsequent gene duplication, nor is there evidence for positive selection of the protein-coding genes in placental species (35). The evolutionary stability of imprinted genes and robustness of their functional networks means that interacting hubs can provide for compensatory actions through common downstream genes. Thus, minor errors can be absorbed and robustness prevails in the functionally complex biological systems they develop. Such error avoidance is essential for imprinted genes that are intergenerationally coexpressed in development (placenta/brain), the interactions of which are of fundamental significance to the survival of both generations.

Many of the downstream genes that are regulated *in cis* by imprinted genes are themselves monoallelically expressed. ENCODE data (36), identified a subnetwork of monoallelically expressed genes that comprised maternal or paternal specific networks. Within each "allelic effect network" those genes regulated by increasing numbers of transcription factors tend to be under stronger negative selection, whereas genes tolerant of loss-of-functions are under weaker negative selection. Genomic imprinting has thus become a coordinator of coadapted gene expression, a viewpoint that has recently been given strong theoretical support (37). These kinds of network interaction often favor the evolution of genetic coadaptation where beneficially interacting alleles evolve to become coinherited.

Histone Acetylation and Methylation. In genomic DNA, nucleosomes comprising ~146 nucleotide bases tightly associated with histone proteins are linked to reduced ease of

transcription. Covalent modifications of the N-termini of histones haven been hypothesized (38) to form an epigenetic "histone code" that is read by other nuclear proteins to facilitate or repress transcription of nearby genes. Thus, histone variants can be "written," "read," and "erased" as epigenetic marks during neural development. Although the study of histone variants and their posttranslational modifications have largely been pursued in nonneural tissue, certain *H3.3* mutations have been reported as highly specific to pediatric gliomas (39), and histone modifications were drawn into neural development during this meeting. That is, in this issue of PNAS, Noh et al. (40) report the "reading" of a combinatorial histone modification, H3K9me3S10ph in postmitotic neurons and relate it to periods of heightened activity.

Retrotransposons. Retrotransposons are selfish genetic elements that may be used to drive evolution, but which may also create serious errors in neural development (Rett syndrome) and somatic development (cancer). The evolutionary impact of retrotransposons has depended on genetic mechanisms to control their stability (41). The epigenetic inheritance of retrotransposon control over gene expression probably reflects their predisposition for DNA methylation. Trim28 is a key player in silencing of endogenous retrotransposons (42), and together with ZFP57 controls levels of methylation safeguarding the transcriptional dynamics of early embryos.

The resulting resistance of retrotranspons to reprogramming leads not only to transgenerational epigenetic effects, but in some cases to the parent of origin effects seen in genomic imprinting (43). It has been suggested that the origins of genomic imprinting were based on retrotransposon insertion, though to date this has been shown to be the case only in one imprinted gene [Ras protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*)]. Imprinting of the *Rasgrf1* locus depends on a noncoding RNA and PIWI interacting RNA (piRNA). A retrotransposon within the RNA is targeted by piRNAs, which directs the sequence specific methylation of *Rasgrf1* (44). Recent evidence indicates that microRNAs were initially formed from transposable element sequences (45). Retrotransposition has also resulted in the imprinting of other genes that are imprinted (*Ipp5p*, *Nap115*, *Mcts2*, and *U2af1-rsl*), which are expressed in the brain (46).

Molecular mechanisms whereby transgenerational inheritance of transposons results in changes to DNA methylation that extend into closely located epialleles have been proposed to result in transgenerational transmission in plants (47). A number of studies in mammals have also found an association between phenotypes and silencing of transposable elements, such as that which occurs in the Agouti mouse. In these animals, transcription resulting from a retrotransposon insertion 100-kb upstream of the *Agouti* gene causes ectopic expression of this gene, leading to yellow fur, obesity, and diabetes (48). The distribution of yellow-fur phenotype differs according to a maternal epigenetic effect, not resulting from the environment but because of incomplete erasure of epigenetic modification when a silenced allele is passed through the female but not male germ line. The coat color of Agouti mouse progeny can be modulated by a diet rich in "methyl donors," but this is lost by the third generation, and is neither stable nor transgenerational (49). However, this same genetic locus has been shown to be under rapid adaptive selection for coat color, raising the possibility that some haplotypes may be prone to epigenetic variation (50).

Elevation of L1 transposons is thought to induce meiotic prophase 1 defects, including oocyte aneuploidy and potential embryonic lethality (51, 52). It is well established that attrition of more than 60% of oocytes in meiotic prophase occurs before birth (53–58). Fetal oocyte attrition may therefore serve to select "healthy" oocytes with limited retrotransposon activities that are best suited for normal development of the next generation (59).

The brain is an important target for retrotransposon mobilization. The line1 promoter has binding sites for the YY1 and sex-determining region Y-box 2 (Sox2) neural transcription factors. Line1 sequences are usually transcriptionally repressed as a result of their methylation in primordial germ cells. However, Muotri et al. (60) reported L1 RNAs to be present in neural progenitor cells derived from the hippocampus, which is congruent with the findings that L1 is hypomethylated in the developing brain. L1 expression has been shown to be repressed in neural stem cells by expression of Sox2 transcription factor and transcriptionally silenced by DNA methylation. Recent studies have shown that neuronal progenitor cells derived from tissue of patients with Retts syndrome and carrying methyl-CpG-binding protein (*MeCp2*) mutations have increased susceptibility to L1 retrotransposition (61). Coufal et al. (62) found high levels of MeCp2 associated with the L1 promoter modulating its developmental activity, but the DNA methyl binding protein 1 did not affect L1 retrotransposition. L1 insertions may be present in only a subset of neurons, thereby producing L1 mosaicism in the brain. Because of L1 mobilization in the later phases of neurogenesis, each neuron may be genetically unique with regard to the cohort of loci containing somatic L1 insertions. This is especially pertinent to the hippocampus, where neurogenesis continues from the subventricular zone into adulthood. Such mosaicisms can produce genetic diversity among subpopulations of neurons. Importantly, LINE1 germ-line insertions are rarely found in regions where they generate a deleterious phenotype because of strong selection against such mutations during evolution. However, the misregulation of mobile elements has been found in the neural disorders of Rett syndrome and schizophrenia (63).

Epigenetic Reprogramming of Neurons. The primordial germ cells of the female fetus undergo demethylation reprogramming for the next generation, isolated from the variance of the outside world in a stabilized in utero environment, as does the male germ line after postfertilization in the zygote (23). In addition to the early phases of methylation reprogramming that act on genomic imprinting and genes that are coadapted for developing the hypothalamus and placenta (64), certain genes that are destined to form the neocortex have a late phase of reprogramming in the postnatal period (65). Indeed, the major part of neocortical brain development occurs postnatally, and its functioning is designed to adapt to the social and physical environment. Unlike germ line cells, cortical neurons require a capacity to reprogram by demethylation/methylation throughout adult life. Demethylation (Tet1) occurs during the process of hippocampal learning and memory (66) and during adult neurogenesis, which impacts on the olfactory system (67). Learning and memory require neuronal activity, which can strengthen synaptic connections and weaken other synaptic connection strengths through a process of synaptic plasticity. The signaling events triggered by synaptic activity involve Ca^{2+} entry to the neuron via a range of neural receptors (NMDA and GABA receptors featuring predominantly), leading to changes in neurotransmitter release and epigenetic changes to DNA methylation, acetylation, and hydroxymethylation. Through these epigenetic changes, neurons gain high plasticity to integrate and store new information. Inhibition of DNA methyltransferases (Dnmt) disrupts long-term potentiation in the hippocampus and alters methylation within the promoter regions of *Reelin* and brain-derived

neurotrophic factor (*BDNF*), two hippocampal genes engaged with synaptic plasticity (66).

Just as Tet1, -2, and -3 methylcytosine dioxygenases play an integral role in the reprogramming of the embryonic germ line, their function is crucial to neurons and the mechanisms underpinning learning and memory (68). Tet-mediated demethylation is found at its highest levels in the brain and is thought to influence both passive demethylation by acting on the Dnmt enzymes and to serve as an intermediary in active demethylation. Tet1 has been shown to promote DNA demethylation in the adult brain at activity-dependent synapses in the hippocampus (69).

Analysis of *Tet1* knockout mice demonstrated down-regulation of hippocampal genes engaged with neural activity (*Npas4*, *c-fos*, and *Arc*), accompanied by impaired memory extinction and abnormal long-term depression (70). *Tet1* mutant mice have also been shown to have impaired short-term learning and memory (71), whereas animals overexpressing Tet1 in the hippocampus have long-term memory impairment (71). No abnormalities were revealed in the developing brain, hippocampal neurogenesis, or neuronal differentiation of *Tet* knockout mice, suggesting a level of redundancy across the methyl cytosine dioxygenases.

A number of genes that are destined to be expressed in the brain escape demethylation in the germ line (25). Such genes are mainly associated with repeat elements, but also include neurally functioning genes. This finding would suggest the possibility for neural-specific expression of genes that undertake demethylation at a later phase in the brain. This would meet the need for conserving methylation into the later stages of neocortical development when postmeiotic cell division and demethylation coincide with up-regulation of the base excision repair pathway (72). Such events would serve to protect the brain against cumulative genetic damage.

Certain regions of the brain (hippocampus, olfactory bulb) continue neurogenesis in the adult brain. Overexpression of Tet3 has been shown to disrupt the successful anatomical integration of the newly generated olfactory receptor neurons (67). The continuous renewal of these sensory neurons is required to maintain neural plasticity in the olfactory system, the most important sensory system for the majority of mammals. Because continual exposure of chemoreceptor neurons to environmental toxins results in their death, their regeneration and replacement throughout life is essential to continue chemosensory function. This regeneration of the receptor neurons also requires some reorganization of neurons in the circuitry at the initial relay in the olfactory bulb. Tet3 action is therefore important to ensure the functional integration of these receptor neurons to sustain sensory recognition.

Neural Development and Neurodevelopmental Disorders

The in utero development of the brain comprises the formation of thousands of unique cell types, each one with its own unique set of functional and molecular properties. This complex developmental event is orchestrated first by gastrulation (which specifies the neuroectoderm) and then by a set of overlapping gradients of signaling molecules. Of these, the most prominent is sonic hedgehog secreted by the notochord, which specifies dorsoventral identity, but Wnt, BMP, and FGF signaling pathways all play major roles in the process as well (73–81).

In addition to the patterning of these gradients, many neurons are specified in regions of the brain distant from where they will reside, and have to undergo a complex migration process, following local cues to arrive to their destination (82).

Double-Stranded RNA-Specific Endoribonuclease (Drosha)/DiGeorge Critical Region 8. Because of the sheer volume of events and cell types, the epigenetics of neural development are difficult to catalog and elucidate. This task, however, is medically crucial, as deviations from normal developmental processes can lead to the personal anguish of a variety of neurodevelopmental disorders, each of which can cost, over a lifetime, millions of dollars per child. Some progress is being made, starting with the mechanisms that cause neurodevelopmental defects of conditions such as Rett's syndrome (83), which is epigenetic in origin. MeCP2, a methylated DNA binding protein, is recognized as a transcriptional repressor, dysfunctions of which lead to the neurodevelopment disorders of Rett syndrome and autism spectrum disorder (84). Recently, MeCP2 was found to be involved in posttranscriptional gene regulation by repressing the processing of nuclear microRNAs through involvement of the Drosha/Dgcr8 complex. MeCP2 binds directly to Dgcr8 (DiGeorge critical region 8), a key component of the nuclear miRNA processing pathway and interferes with the assembly of Drosha/Dgcr8 (84). Interaction of DGCR8 with MeCP2 (84, 85) is independent on the DNA binding domain of MeCP2, but controls microRNA processing via the direct interaction with Dgcr8. One particular microRNA known to be regulated by Dgcr8 is miR134, which regulates the expression of *Nanog* and *BDNF* (86).

Noncoding RNAs are gaining recognition in the context of brain development and psychiatric disorders, and are represented in this Sackler Colloquium by the paper of Goff et al. (87). Chromosomal 22q11.2 deletion is the strongest known risk factor for schizophrenia (88). A primary candidate gene is *Dgcr8*, which encodes a component of the RNA microprocessor complex (Drosha/Dgcr8), essential for microRNA biogenesis. Mir185 in particular is the most notable down-regulated miRNA in both the prefrontal cortex and hippocampus, brain areas that are focal to schizophrenia research (89). Haploinsufficiency of *Dgcr8* in a mouse model revealed synaptic *SERCA2* [sarco (endo) plasmic reticulum Ca^{2+} ATP-ase] overexpression, a finding that parallels the elevated levels of Ca^{2+} ATP-ase found in schizophrenia brains (90).

Autism. Autism spectrum disorders present a significant and urgent challenge. Now representing more than 1 in every 100 children, diagnoses of autism depend on abnormal social behaviors, compulsive repetitive movements, and language disorders. Because of the overwhelming evidence that various forms of prenatal stress contribute to autism (91–93), epigenetic modifications provide an obvious set of possible mechanisms for the spectrum of autism-like disorders.

In some children with autism, mutations in the genes encoding subunits of SWI/SNF-like complexes, ATP-dependent chromatin remodelers seem to provide an epigenetic basis for the disease (94). Similarly, Noonan and colleagues have reported that mutations in *CHD8* (chromodomain/helicase-DNA binding protein) are nearly always autism-causing (95). In addition, *CHD8* haploinsufficiency results in down-regulation of multiple genes involved in early brain development. Mutations in many of these genes are associated with autism (96). BDNF, acting through its effects on several miRNAs by affecting the Lin28/lethal-7 (Let-7) axis during development (97), exemplifies the kind of local chemical influence whose dysregulation could contribute to autism. These data comprise the vanguard of a large number of potential investigations into the epigenetic mechanisms by which early stress could contribute to the development of symptoms of autism, but much work remains to be done.

In fact, epigenetic phenomena may provide part of the "missing heritability" in autism genetics. Although MZ twins clearly have higher rates of autism diagnoses than dizygotic twins or siblings (98), pure genetics

only accounts for part of that difference. There is a discrepancy: actual heritability is greater than differences accounted for by changes in DNA (99). Consider that the egg and the surrounding cumulus cells provide the first microenvironment that the future twins encounter as they develop. Therefore, MZ twins may share common epigenetic changes, initiated before the embryos become separate from each other. These shared epigenetics of MZ twins could account for part of the heritability of autism.

Potential Usefulness of Human Induced Pluripotent Stem Cells. Much of the research in the epigenetics of neural inheritance has been done in rodent models. Although rodent models are indubitably crucial, the epigenetics of human neurological disorders can be difficult to approach, especially in cases where the genetic basis is unknown. This is especially true for where psychoactive medications are available, such as schizophrenia and major depressive disorder. In such incidents, even investigations of the epigenetics of postmortem tissue may not provide answers as to what causes the disease, as it can be difficult to disentangle any changes that occur from the effects of the psychoactive medication used to treat the disease.

A promising alternative is the recently emergent field of disease modeling in vitro using human pluripotent stem cells derived from fibroblasts of patients suffering from the disorder (100). Recent studies in schizophrenia, Huntington's disease, spinal muscular atrophy, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (100–106) among others (previously reviewed; see refs. 100 and 107) illustrate the potential utility of stem cells in understanding the functional and epigenetic bases for such diseases.

However, potential obstacles remain, of which perhaps the greatest is the microenvironment of cultured cells. Whereas some self-patterning and 3D culture approaches combined with agonists of signaling pathways have proven promising in producing cells with similar properties to those found in vivo (108–110), it has yet to be fully established how the microenvironment of these cultures mimics the microenvironment normally experienced by these cells during development. The answer is likely to be different for each neural subtype. Similarly, glial cells can have tremendous effects on neuronal development and function, and provide another complex variable to be investigated for in vitro modeling (111).

It is also possible to circumvent the technical challenges of replicating the microenvironment of the brain in vitro by transplanting induced pluripotent stem cell-derived neural cells into the brain and by subsequently analyzing their properties in vivo. Many studies have been published illustrating the engraftment of various pluripotent cell-derived neurons into the mature nervous system in rodents and monkeys (112–127). Functional testing has demonstrated that these cells can integrate and function in the organisms, sometimes repairing defects and lesions (113, 114, 120, 122–125). An open question remains about the epigenetics of these grafts. Do they retain an epigenetic memory of their time in cell culture, and how might that affect their function and viability in the long term? Despite this caveat, in vivo transplantation of neurons obtained from stem cells is an excellent tool both for functional testing and addressing biological question.

An additional complication arises from the process of reprogramming adult cells (usually fibroblasts from patients) to a pluripotent state (128–130). Whether achieved by somatic cell nuclear transfer or by viral transduction, reprogramming, by definition, is a cause of epigenetic modifications. This presents an additional challenge for modeling the epigenetics of environmental stresses, such as those that occur in autism, as the stresses themselves, and how they are experienced by particular neural cells during development, must be very well understood in order for in vitro modeling to proceed. Nevertheless, there is cause for optimism that cell culture-based systems will eventually provide insights into phenotypes and epigenetic perturbations found during neurodevelopmental events in vivo. A combination of postmortem studies and in vitro modeling could help to disentangle cause and effects in the epigenetics of neurodevelopmental disorders.

Intergenerational Coadaptation

In behavioral biology there is a small but growing group of phenomena in which a single biological process, when modulated in the mother's brain to affect a given class of behaviors, also affects that same class of behaviors through corresponding changes in the baby's developing brain. A behavioral example would be the quality of maternal care factors that can, in some cases, result in poor maternal behavior by the next generation of female offspring (13). A genetic example would come from work with the imprinted *PEG3* gene. Fluctuations in the expression of PEG3 in the developing placenta are coordinated with this same gene's expression in the next generation's developing hypothalamus. Such intergenerational coadaptation occurs in the fetal hypothalamus as it commences early in utero development. At the same time, the fetal placenta, by way of hormone production, engages with the hypothalamus of the mother (previous generation). A number of imprinted genes are coexpressed in the developing hypothalamus and placenta at this stage, ensuring that the developing fetal hypothalamus expresses the same allele that functioned in the development of the maternal hypothalamus of the previous generation. This intergenerational action (fetal placenta/maternal hypothalamus) provides a template on which selection pressures have acted to ensure successful mothering. By selecting for intergenerational functional coadaptation involving genes that coregulate development of the placenta and hypothalamus, evolutionary progress in the direction of successful mothering has been faster than if the same gene had been required to operate independently in each of these structures in each generation. Thus, the success of developing a hypothalamus that is intergenerationally responsive to hormones from the fetal placenta thereby ensures good mothering by the next generation of daughters (64).

A number of coexpressed imprinted genes contain clusters of small noncoding RNAs, which are regulated by the Drosha/Dgcr8 complex, which is also expressed in the developing brain and placenta. Noncoding RNAs are gaining recognition in the context of neural development and psychiatric disorders, as well as dysfunctions that occur in placental development. The imprinted C19 cluster of miRNAs is associated with pregnancy complications. Different miR members are important for placental primary trophoblast development (miR141, miR21) and for extravillous trophoblast invasion of the maternal decidua and myometrium (miR519) (131). Others are expressed in exosomes released from primary villous, which endow nontrophoblastic cells with resistance to viruses (132). Thus, the C19 microRNA cluster is targeted by Dgcr8 to regulate this exceptionally large imprinted microRNA cluster for cell specific placental development (133).

An important consideration in the context of coadaptive brain and placental development for ensuring good mothering is how the developing male brain ensures diversion from the maternalistic trajectory. Sex-determining region on the Y (*SRY*), the male sex-determining gene, has evolved only in placental mammals and is not a feature of egg-laying monotreme mammals. Interestingly, *SRY* is a hybrid gene of *Dgcr8* and sex-determining region Y-box 3, (*Sox3*) (134). Dgcr8 has a notable role in the regulatory control of microRNAs in both brain and placental

development, whereas Sox3 is fundamental to development of the hypothalamus. The hypothalamic nuclear groupings that regulate male sexual behavior have similar developmental origins to those hypothalamic regions responsible for maternal behavior in females, but have undergone masculinization by testosterone produced in the fetal Leydig cells. The testosterone-producing Leydig cells are also under developmental control of the sexually dimorphic Let7 family of miRNAs (*miR140-3p*, *miR140-5p*, *miR378*) (135). Noncoding RNAs are clearly important in multiple aspects of brain, placental and testes development thereby making available the potential for coordinated developmental timing across different but functionally integrated systems.

Some Outstanding Questions

Finally, the following is a list of some outstanding questions emanating from the Sackler Colloquium:

i) What are the roles of DNA methylation, including consideration of CpG islands and their "shores"? Old stereotypes are currently being questioned.

ii) What is the temporal order of histone modifications associated with any given neurally expressed gene, and their combinatorial logic of the relevant "writers," "readers," and "erasers"?

iii) What are the relations among different epigenetic marks? For example, can DNA methylation regulate certain histone modifications and/or the reverse?

iv) Does transcription factor binding actually control histone modifications, as has recently been suggested (136)?

v) What is the biological significance of transposon expression in the CNS? Although their suppression following behavioral stress was emphasized in one paper (14), positive implications for expanded opportunities for neuroplasticity have also been pointed out (63, 137).

vi) What are the roles of long noncoding RNAs in the modification of neuronal nuclear architecture (138)?

vii) A large number of behavioral epigenetic studies attempt to correlate epigenetic marker changes (e.g., acetyl histone H3) at global levels and in mixed populations of cells with phenotypic changes. Specific changes at specific gene levels and at single cell levels correlating with behavioral changes remain largely unknown.

The initial purpose of the meeting, to foster cross-talk between the chemistry of epigenetics and current neuroscience, was further served by refs. 139–143. All presentations made evident the fact that the variety and subtlety of the chemical modulations of epigenetics would likely be sufficient to provide mechanisms for nervous system modulations as currently understood.

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