

PROCEEDINGS
FROM
UNDERSTANDING
THE BIOLOGY
OF SEX DIFFERENCES

SCIENTIFIC ADVISORY MEETING

SEX BEGINS
IN THE WOMB

MARCH 1, 2002
CROWNE PLAZA CABAÑA
PALO ALTO, CA

JOINTLY SPONSORED BY



SOCIETY FOR
WOMEN'S HEALTH RESEARCH



STANFORD
UNIVERSITY
SCHOOL OF MEDICINE

CENTER FOR RESEARCH ON WOMEN'S HEALTH
& REPRODUCTIVE MEDICINE AND THE
DEPARTMENT OF GYNECOLOGY & OBSTETRICS



UNDERSTANDING
THE BIOLOGY
OF SEX DIFFERENCES

SCIENTIFIC ADVISORY MEETING

**SEX BEGINS
IN THE WOMB**

MARCH 1, 2002
CROWNE PLAZA CABAÑA
PALO ALTO, CA

JOINTLY SPONSORED BY



SOCIETY FOR
WOMEN'S HEALTH RESEARCH



CENTER FOR RESEARCH ON WOMEN'S HEALTH
& REPRODUCTIVE MEDICINE AND THE
DEPARTMENT OF GYNECOLOGY & OBSTETRICS

©2002

Society for Women's Health Research
1828 L Street, NW, Suite 625
Washington, DC 20036
Phone: (202) 223-8224
Fax: (202) 833-3472
www.womens-health.org

"Sex Begins in the Womb" and these meeting proceedings were supported by an unrestricted educational grant from Berlex Laboratories, Inc., an affiliate of Schering AG.

These meeting proceedings were written by Nancy Evans for the Society for Women's Health Research in cooperation with the speakers and Society staff members Stacey Fannon and Regina Vidaver.

TABLE OF CONTENTS

Continuing Medical Education (CME) Information	5
Imprinting and X-Inactivation	6
The Biology of the X Chromosome: Compensating for Sex Differences, Huntington F. Willard, Ph.D.	
Biological Consequences of Imprinting Evolution, Randy L. Jirtle, Ph.D.	
IGF2 Imprinting, DNA Methylation and Cancer, Andrew R. Hoffman, M.D.	
Intrauterine Environment	14
The Intrauterine Position Effect, John G. Vandenberg, Ph.D.	
Effects of Fetal Exposure to Natural and Exogenous Steroids, Frederick S. vom Saal, Ph.D.	
Fetal Stress Responses: Prenatal Stress and Sexual Differentiation, Vivette Glover, Ph.D.	
Fetal Origins of Adult Diseases: A Hypothesis, Daniel T. Lackland, Dr.P.H.	
Congenital Diseases.....	21
Sex Differences in Mental Retardation, Charles J. Epstein, M.D.	
Females are Unique Mosaics: The Path from Muscular Dystrophy to Recurrent Pregnancy Loss, Cheryl A. G. Scacheri, M.S.	
Congenital Adrenal Hyperplasia: Prenatal Androgens and Behavioral Sex Differences, Sheri A. Berenbaum, Ph.D.	
Sex and the Developing Brain	27
Cellular Mechanisms Underlying Development of Sexually Dimorphic Neural Pathways, Richard B. Simerly, Ph.D.	
Mechanisms of Steroid Mediated Brain Differentiation, Margaret M. McCarthy, Ph.D.	
Effects of Maternal/Fetal Interaction on Neural Development, Christine K. Wagner, Ph.D.	
Speaker Biographies	31
Sex Begins in the Womb Planning Committee	34
About the Society for Women's Health Research	35
Staff	
Board of Directors	
CME Post Test	38

CONTINUING MEDICAL EDUCATION (CME) INFORMATION

DISCLOSURE

In accordance with the Accreditation Council for Continuing Medical Education (ACCME) Standards for Commercial Support, the speakers for this course have been asked to disclose to participants any significant relationships with commercial entities that are either providing financial support for this program or whose products or services may be mentioned during their presentation. None of the speakers disclosed a conflict of interest.

TARGET AUDIENCE

This material is intended for researchers and clinicians in the fields of gynecology and obstetrics, maternal/fetal medicine, pediatrics, endocrinology, IVF, genetics, neuroscience, reproductive biology, molecular biology, developmental biology, sex-based biology, and women's health.

ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the ACCME through the joint sponsorship of Stanford University School of Medicine and the Society for Women's Health Research. Stanford University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians.

Stanford University School of Medicine designates this educational activity for a maximum of 2 hours of Category 1 credit toward the AMA Physicians' Recognition Award. Each physician should claim only those hours that he/she actually spends in the educational activity.

Material release date: January 2003

Material expiration date: January 2005

EDUCATIONAL OBJECTIVES

After reviewing the material contained within, the participant should be able to recognize and describe causes and consequences of sex differences originating during prenatal development. Participants are expected to use this knowledge to conduct sex-specific research and/or to provide sex-specific health care, ultimately leading to better health and more personalized disease prevention, diagnosis, and treatment.

To obtain CME credit for reviewing this material, see page 38.

IMPRINTING AND X-INACTIVATION

Genomic imprinting and X-inactivation are two epigenetic mechanisms by which mammals selectively silence one or the other member of certain allelic pairs. Imprinting occurs in both males and females and regulates gene activation according to parent-of-origin inheritance. In contrast, X-inactivation is a female-specific biochemical mechanism in mammals that silences most—but not all—genes on one or the other X chromosome in each cell, as a form of dosage compensation for X-linked genes.

Both genomic imprinting and X-inactivation processes are characterized by: hypermethylation, modified histone tails and histone variants, and late replication of the silenced regions in the S phase of the cell cycle. In the opening session of the conference, speakers explored the fundamental biology of X-inactivation, the evolution of genomic imprinting, and the medical and biological consequences of imprinting the *IGF2* and *IGF2R* genes.

THE BIOLOGY OF THE X CHROMOSOME: COMPENSATING FOR SEX DIFFERENCES *Huntington F. Willard, Ph.D.*

The process of X-inactivation begins about the time of implantation of female embryos. The selection of which chromosome becomes inactive is usually random, making females cellular mosaics, with some cells expressing alleles from the paternally inherited X chromosome and other cells expressing alleles from the maternally inherited X chromosome.

Huntington F. Willard of the University Hospital of Cleveland and Case Western Reserve University School of Medicine, explained that the

onset of X-inactivation is controlled by the *Xist* gene. *Xist* is expressed only from inactive X chromosomes in somatic cells, and is a key component of the X-inactivation center (XIC). In the human, the XIC resides in the proximal long arm of the X chromosome. The cell recognizes that there are two X chromosomes by “counting” copies of the XIC in that cell. Once two X chromosomes are counted, *Xist* is activated, and an RNA molecule is produced which “coats” one of the X chromosomes, ultimately rendering that X chromosome almost completely genetically inactive. “This is over a stretch of some 150 to 160 million base pairs of DNA,” Willard explained. “From a molecular perspective, X-inactivation is a biological tour de force—somehow spreading the silencing effects north and south along one X chromosome without influencing the other X chromosome in the same cell.” Certain genes, however, escape X-inactivation. How this escape occurs is unknown.

To determine the X-inactivation profile of the human X chromosome, Willard and his colleagues evaluated the expression of nearly 500 X-linked genes in a panel of mouse/human somatic cell hybrids containing a human inactive X, but no active X. They also evaluated a collection of 40 different human cell lines that show complete non-random inactivation as monitored by evaluation of expressed X-linked polymorphisms.

Thus far, the data reveal at least five different types of genes that show distinct behavior in response to X-inactivation. First, the majority of genes in female cells are expressed only from the active X chromosome, while those genes on the inactive X chromosome undergo DNA methylation and chromatin condensation, and are fully inactive.

Second, the *Xist* gene itself behaves in exactly the opposite way. *Xist* is well expressed from the

inactive X chromosome in female somatic cells but not at all expressed from the active X chromosome. It is also not expressed from the X chromosome in male somatic cells.

Third, between 15 and 20 percent of the genes studied by Willard and colleagues escape inactivation and are well expressed from the inactive X as well as the active X chromosome. These genes represent a fundamental difference in terms of the level of gene output between male and female cells: they are expressed up to two-fold in females compared to males. “This was the single biggest surprise in the field,” Willard said. “If there are hundreds and hundreds of genes on the X chromosome expressed in two doses in females instead of one, that opens the door for fundamental biological and biochemical differences between the sexes.”

Fourth, another class of genes shows variable inactivation. This variability can occur either between females in the population or between tissues within the same individual. Further study is needed on tissue heterogeneity for a growing number of genes on the X chromosome. “The more we look, the more examples of this we find,” Willard continued, “and that really sets the stage for an enormous degree of variability, not only between males and females as a group but among females in the general population. There’s a possibility for major phenotypic variation in the female population that just doesn’t exist in males.”

Fifth, some genes are unstably inactivated, or variably activated over time. In this case, a gene might be subject to X-inactivation early in development, and expressed only in a single dose, but later on in development, or in certain tissues, that same gene will become active and be expressed in two doses instead of one. This phenomenon has been observed in tissue culture cells but further study is needed to determine whether unstable X-

inactivation happens *in vivo*.

Examination of approximately 100 genes in the short arm of the X chromosome, especially in Xp22, shows that the profile of gene inactivation is decidedly non-random. Roughly three-quarters of the genes tested escape inactivation. However, only about 25 percent of all X-linked genes have been assayed for inactivation. Thus a comprehensive survey is underway in collaboration with the Human Genome Project. A complete X-inactivation profile will offer new insights into clinical cytogenetics, medical genetics and genetic counseling, as well as the nature of genetic contributions to sexual dimorphism.

Bioinformatics studies by Laura Carrel, a member of Willard’s group, suggest that X-inactivation is controlled both regionally and locally. Regional control appears to be mediated by long interspersed repetitive DNA elements (L1 elements). The presence of L1 elements—which are in different places in different genes (upstream or downstream, for example—is tightly correlated with the ability of that gene to undergo X-inactivation. Genes lacking in these elements, or other similar elements, are more likely to escape X-inactivation. Local control may reflect the existence of either chromosomal or gene-specific signals that can override the X-inactivation signal.

Willard reported on the work of Brian Chadwick, a member of his laboratory, in identifying variants of core histones on active and inactive X chromosomes and their role in silencing chromatin. The typical core histones that form the fundamental unit of chromatin include H2A, H2B, H3 and H4. These histones have variable lengths but quite short tails at either the amino-terminus or carboxy-terminus. Modifications such as acetylation and methylation occur in the histone tails, affecting compactness of the nucleosome and how tightly the DNA is wrapped around it.

Variants of the core histone H2A are proving to be a fruitful area of research. One of the first histone H2A variants studied by Chadwick is H2A-Bbd (Barr body deficient), which is a component of nucleosomes on the active X chromosome but absolutely avoids the inactive X chromosome. Another H2A variant, macro H2A, associates with the inactive X chromosome. Macro H2A is completely coincident with the *Xist* RNA molecules on the same inactive X chromosome during interphase, suggesting that a direct complex might form between these two molecules.

During metaphase, macro H2A appears to be confined to distinct bands on the inactive X chromosome, not the entire X chromosome. One of those regions is coincident with the XIC. Why other regions attract macro H2A rather than conventional core H2A requires further study.

Willard concluded by stating that “the genes themselves and the genomic elements surrounding those genes, both for X-inactivation and for imprinting, are part and parcel of this silencing mechanism, and clearly set up the two different parts of the genome that are either well expressed or inactivated.”

BIOLOGICAL CONSEQUENCES OF IMPRINTING EVOLUTION

Randy L. Jirtle, Ph.D.

Genomic imprinting is a parent-of-origin-dependent form of gene regulation and a notable exception to Mendelian laws of inheritance. Imprinted genes play a critical role in embryonic growth and behavioral development as well as in cancer susceptibility.

According to Randy L. Jirtle of Duke University Medical Center, why imprinting developed is the subject of active debate among scientists. The most actively debated theory and the one the data

seem to support best is the parent-offspring conflict theory developed by David Haig of Harvard University and Christopher Graham of the University of Oxford in 1991 when the first two imprinted genes, for insulin-like growth factor II (*IGF2*) and the *IGF2* receptor (*IGF2R*), were identified. These two proteins have counteracting growth effects since *IGF2* stimulates growth, whereas *IGF2R* inhibits it by binding *IGF2* for degradation.

This parental conflict theory suggests that imprinting holds no advantage for mammals, but is merely a vestige of a genetic battle between the sexes to control the amount of nutrients that the offspring extract from the mother. Such a conflict would have occurred in polyandric animals, in which the female has the potential to be impregnated by multiple males. The theory predicts that the mother’s copy of a given gene would be turned off for any imprinted gene that promotes growth, whereas the father’s copy would be turned off for any imprinted gene that inhibits growth. A larger offspring will have a survival advantage, making it more likely to pass on the father’s genes; an overly large offspring imperils the mother, thus limiting the survival advantage of larger fetal size. Thus far, imprinted genes directly tested for their effects on growth have fit this theory quite well.

The first evidence that the male and female genomes were not equivalent came from the pioneering work of James McGrath and Davor Solter of the Wistar Institute and Azim Surani and his colleagues at the University of Cambridge in the mid-1980s. These researchers enucleated a mouse egg, and inserted one set each of maternal and paternal chromosomes, which led to normal development, normal sized embryos, yolk sacs and trophoblastic placental cells. When they did the same experiment with two copies of maternal chromosomes and no paternal chromosomes, the

embryo was smaller than normal, the yolk sac was near normal, but there was no placental growth. In a third experiment, two copies of male chromosomes were inserted with no insertion of female chromosomes, which resulted in no embryonic growth, a normal yolk sac, and huge placental growth. This was the first indication that genes expressed only from the mother's copy are required for embryonic growth, and genes expressed only by the father's copy give rise to and control the growth of the placenta.

Imprinted genes not only affect embryonic growth, but carcinogenesis as well, because their functional haploid state makes them vulnerable to either inappropriate inactivation or overexpression. Jirtle used the analogy of a twin-engine jet: "You do not need both engines to fly, but you feel comfortable because you know if one engine goes out, you still have one that allows you to land, whereas imprinting means you are flying all the time with a solo engine."

To trace the evolution of imprinting, Jirtle discussed three groups of mammals: monotremes (duck-billed platypus and echidna), marsupials (kangaroo and opossum), and eutherians (humans and other placental mammals). He cited the work of Keith Killian, a graduate student in his laboratory, who isolated the *IGF2* and *IGF2R* genes from the platypus, echidna and opossum. Killian found that neither gene is imprinted in the most ancestral mammals, the egg-laying monotremes, but that both genes are imprinted in marsupials. Thus, imprinting evolved with the advent of mammalian live birth. Interestingly, while *IGF2* is imprinted in all viviparous mammals (i.e., mammals that reproduce by live birth), *IGF2R* appears to be imprinted only in non-primate viviparous mammals. Jirtle found no evidence of *IGF2R* imprinting in humans, either during embryogenesis or postnatally.

This finding is in stark contrast to a report by Constantin Polykronakos and his colleagues at McGill University who published evidence that *IGF2R* imprinting is a polymorphic trait in humans. Whether a small population of humans exists that are imprinted at the *IGF2R* locus is a critical issue to resolve because in 1995, Angus De Souza, while a postdoctoral fellow in Dr. Jirtle's laboratory, demonstrated that *IGF2R* is a tumor suppressor gene. Thus, individuals with only one active copy would be more susceptible to cancer formation than those with two copies.

Taking another tack, Jirtle and colleagues studied the situation from an evolutionary perspective, using pro-simian (ringtail lemur), which are at the base of the primate family and near-primate (the tree shrew and flying lemur) models. They found that, similar to humans, *IGF2R* is not imprinted in any of these animals. This indicates that *IGF2R* imprinting was lost in a common ancestor to primates some 75 million years ago. This unique finding means that rodents, cows, pigs, sheep, and marsupials have only one functional copy of *IGF2R* while humans have two functional copies. This marked divergence in *IGF2R* imprinting affects species' susceptibility to cancer, and therefore has important implications for cancer research, since mice and rats are often used as surrogates for humans.

Mouse studies have shown that the elimination of the *IGF2R* protein results in large offspring syndrome, a lethal mutation characterized by large size (50 percent larger than normal), immature lung development, polydactyly, enlarged hearts and abnormal bone development. These abnormally large offspring die either just before or after birth. Large offspring syndrome occurs frequently in cloned mice and livestock animals. Jirtle speculated that because humans and other primates do not have the machinery required to imprint *IGF2R*, "it might be easier to clone

humans than non-primate mammals since they would be less prone to develop large offspring syndrome.”

Loss or overexpression of imprinted genes also can affect behavior, as Surani and his colleagues discovered when they knocked out paternally expressed genes 1 and 3 (*PEG1* and *PEG3*). Female mice deficient in either of these genes abandoned their offspring. Thus, both *PEG1* and *PEG3* appear to code for nurturing behavior. Surprisingly, the maternal behavioral effects of these genes are inherited from the father since only the male copy of these “nurturing genes” is expressed. Susan Murphy, a postdoctoral fellow in Jirtle’s laboratory, recently demonstrated that *PEG3*, like *PEG1*, is imprinted in humans during development and throughout adulthood. These provocative findings suggest that nurturing behavior in humans could also be significantly affected by a single mutation in either of these genes, although such mutations have not been identified.

Jirtle outlined three different types of imprint control. In the first type, the maternal copy of the gene is turned off simply by methylation of the promoter region. This simple imprinting regulatory mechanism does not appear to be used to imprint paternal alleles. An explanation for this dramatic sex difference in imprinting regulation is that the paternal genome seems to be actively demethylated soon after egg fertilization. Thus, more elaborate and convoluted imprinting mechanisms involving antisense RNA or reciprocal imprinting of juxtapositioned genes, utilizing both enhancer and repressor elements are needed to turn off the paternal copy of the gene. Citing work by Wolfgang Mayer and his associates at the Max Planck Institute of Molecular Genetics, Jirtle explained that within 8 hours after fertilization, the paternal genome is decondensed and demethylated, whereas the maternal genome is protected from these effects.

Were the primordial imprinting mechanisms less complex than those presently found in placental mammals? Jirtle’s lab is using an evolutionary approach to answer this fundamental question. “Evolution is a biologist’s Hubble telescope,” he said. “It allows us to look back in time, but it doesn’t cost a billion dollars and hopefully it’s not myopic.” Jirtle is presently performing phylogenetic comparisons of orthologous domains in the platypus, opossum, and human genomes. Genetic sequences in these domains that are conserved in the imprinted marsupials (opossum) and placental mammals (human), but are divergent in the non-imprinted monotremes (platypus) are likely to be mechanistically involved in regulating genomic imprinting.

Jirtle’s lecture concluded with a quote from Albert Einstein who said, “God is subtle, but he is not malicious.” Jirtle said that he is now devoting his scientific energies to identifying those subtle changes that resulted in the evolution of imprinted genes over 150 million years ago, and consequently established unique genetic targets in our genome by which environmental exposures can potentially alter our susceptibility to both developmental disorders and cancer.

***IGF2* IMPRINTING, DNA METHYLATION AND CANCER**

Andrew R. Hoffman, M.D.

The final presentation in the session on imprinting and X-inactivation was given by Andrew Hoffman of VA Palo Alto Health Care System and Stanford University. Hoffman described the role of imprinting and DNA methylation in cancer. Hoffman’s work focuses on the mitogenic peptide IGF2, which stimulates cell division and lymphocyte transformation. IGF2 is part of an extended family that includes insulin-like growth factor 1 (IGF1), insulin, and relaxin, a key hormone in parturition.

The IGF2 protein acts through three different receptors. First, it interacts at the IGF1 receptor in exerting mitogenic and anabolic activities. Second, IGF2 interacts anabolically through the type A insulin receptor, but can also induce hypoglycemia. Hyperexpression of IGF2 leads to potentially fatal hypoglycemia in malignant diseases, such as in malignant sarcoma. Third, IGF2 interacts with IGF2R, which regulates degradation of IGF2.

In the mouse, *IGF2* has three promoters that are homologous to promoters present in the human gene. However, humans have an additional promoter, P1, which is the predominant promoter in the liver, one of the major sites for IGF2 synthesis in the adult. In the human fetus, *IGF2* is always imprinted. This promoter does not exist in any species below primates.

IGF2 is expressed from the paternal allele from all promoters except for human promoter P1, which drives expression biallelically. This was the first example found of promoter-dependent imprinting.

The central nervous system (CNS) offers an example of tissue-specific imprinting in both humans and mice. In the CNS, *IGF2* is usually biallelically expressed from all promoters.

Partial or complete loss of imprinting can result in inappropriate expression of the maternal *IGF2* allele. Hoffman pointed out that biallelic expression would lead to increased cell division and potentially to unregulated growth and neoplasia. He cited the 1993 work from the laboratories of Tony Reeve of the University of Otago, Dunedin, New Zealand and Andy Feinberg of Johns Hopkins University showing that about two-thirds of patients with Wilms' tumor had biallelic expression of *IGF2*. Since then, numerous malignancies in adults and children have been found to exhibit

biallelic expression of *IGF2*, including tumors of the colon, liver, smooth muscle, and adrenal glands. Most of these tumors also synthesize the IGF1 receptor, through which IGF2 exerts its mitogenic activity.

The question of whether IGF2 overexpression is an etiologic factor in tumor formation or merely an unrelated phenomenon in cancer cells has not been definitively answered. However, Hoffman cited a 1993 case report by O. Ogawa and colleagues at the University of Otago, Dunedin, New Zealand suggesting that IGF2 overexpression increases the risk of tumor formation. In the Ogawa study, a child with Wilms' tumor and gigantism had biallelic expression of *IGF2*, not only in the tumor but also in the normal kidney and in the peripheral blood leukocytes. The authors described this as a "constitutional relaxation of imprinting" and suggested that some individuals might have this lack of imprinting from birth, putting them at increased risk for developing tumors.

Hoffman reported on several studies in his laboratory examining paired samples of Wilms' tumor and normal adjacent kidney tissue. In the initial study, they found loss of imprinting of *IGF2* in 6 out of 8 tumors. In 5 of the tumors showing loss of imprinting, the normal adjacent tissue also showed loss of *IGF2* imprinting. These findings lend credence to the idea put forth by Ogawa and colleagues that imprinting happens early in development and affects an individual's risk of cancer.

To determine whether the loss of *IGF2* imprinting affected expression of *IGF2* mRNA, Hoffman's group examined 40 paired samples of Wilms' tumor and normal adjacent tissue. In all cases, they found that *IGF2* mRNA levels were greater in the tumor tissue, with or without loss of imprinting. In normal tissue exhibiting loss of imprinting, *IGF2* mRNA levels were also elevated.

Excessive elevation of *IGF2* mRNA in tumor tissue correlated with loss of imprinting and increased *IGF2* mRNA in the adjacent normal tissue.

Loss of imprinting in the *IGF2* gene is not the only mechanism for hyperexpression of *IGF2* mRNA in tumors. For example, almost all Wilms' tumors that have been studied show hyperexpression of *IGF2* mRNA even without loss of imprinting.

Hoffman pointed out that loss of *IGF2* imprinting also occurs in normal tissue adjacent to other malignancies such as colorectal cancer. He cited the work of Feinberg's group that found loss of *IGF2* imprinting in about half the colorectal cancers examined and, in these patients, loss of imprinting was apparent in the normal adjacent colonic mucosa and in some of the peripheral leukocytes. This study offered further evidence of a constitutional lack of imprinting in some patients, potentially increasing their risk for developing tumors.

DNA methylation is one of the most important mechanisms involved in imprinting. As Hoffman explained, "the human genome contains only 10 percent of the CpG dinucleotides that you would expect to see if all dinucleotides appeared by chance. Instead, there are small regions of DNA called CpG islands that contain the normal expected frequency of CpGs, which are generally protected from methylation." These unmethylated islands are associated with chromatin containing acetylated histones and these modifications are characteristic of highly transcribed DNA. However, some regions of these CpG islands are methylated and are associated with both X-inactivation and silenced alleles.

To study the role of methylation, Hoffman and his colleagues exposed astrocytes cultured from mouse and human brain tissue to the demethylating agent 5-azacytidine or 2-deoxy-5-azacytidine.

Exposure through increasing cell culture passages dramatically enhanced the expression of *IGF2* mRNA driven by promoter P3 in the mouse and driven by promoter P4 in humans. Expression driven by the P2 promoter remained unchanged.

Injection of 5-azacytidine into young animals also thoroughly and randomly disrupted *IGF2* imprinting. In some tissues such as the spleen, imprinting remained; in the lung, a reversion occurred, wherein the normally expressed allele was silenced and the normally silenced allele was expressed. In the heart, biallelic expression was evidenced. The changes were inconsistent from animal to animal, suggesting random loss of imprinting. As Hoffman explained, "Imprinting was completely disrupted and remained disrupted, we believe, through the lifetime of the animal from the exposure to demethylating agents."

Hoffman and his colleagues also looked at how histone acetylation and histone methylation interacted with DNA methylation to initiate or maintain imprinting. They found that histone acetylation increases gene transcription while histone deacetylation represses transcription. Histone deacetylation in the *IGF2* differentially methylated region 0 (DMR0) using trichostatin A, which only deacetylates histones, also decreased the amount of DNA methylation in that region.

These findings have led to a model in which expressed, non-imprinted alleles are associated with acetylated histones, unmethylated DNA, and a relaxed chromatin structure that allows the gene to be expressed. The imprinted allele is associated with unacetylated histones, methylated DNA, and a more compact chromatin structure. According to Hoffman, this model is undergoing intense scrutiny.

Based on their knowledge of methylation and the role of *IGF2* expression, Hoffman and his col-

leagues sought a way to specifically alter *IGF2* expression and thereby decrease the growth of tumors. They devised a methylated oligonucleotide (MON1) that they hoped would block *IGF2* gene expression by hypermethylating the *IGF2* gene at the expressed allele. The researchers hypothesized that the oligonucleotide attracts DNA methyl transferase (DNmt-1). Blocking gene expression would ultimately decrease IGF2 protein, thereby decreasing tumor growth.

After exposing cultured human fibroblasts and mouse astrocytes to either MON1 or phosphate buffered saline (controls) for 24 hours, the investigators found no *IGF2* mRNA in the cells exposed to MON1. Similar results were found using a human hepatocarcinoma cell line that normally overexpresses *IGF2*.

Another experiment using an oligonucleotide without methylation showed no effect on *IGF2* expression, nor did an anti-sense oligonucleotide. As Hoffman explained, “there seems to be a very specific effect to the methylated oligonucleotide. It hones in on the nucleus of these cells and when we inject it in a lipid carrier . . . we can completely inhibit *IGF2* from the liver of normal mice.”

When mice were injected with hepatocellular carcinoma cells and subsequently developed large hepatic tumors, Hoffman and his colleagues treated some of the mice with MON1. In the untreated animals, the tumors continued to grow, doubling in size every 10 days, eventually causing death. In the treated animals, however, two doses of MON1 resulted in a 50 percent decrease in tumor size and longer survival time. A similar experiment using a different methylated oligonucleotide (MON2) also showed dramatic reduction in tumor size and enhanced survival.

As Hoffman concluded, these findings indicate that using pharmacological methods to change DNA

methylation can alter the allelic expression of *IGF2*, both *in vitro* and *in vivo*. By examining how loss of imprinting occurs, it may be possible to develop therapies that will slow tumor growth or cause tumors to re-differentiate by restoring normal allelic expression.

INTRAUTERINE ENVIRONMENT

The developing fetus is exquisitely sensitive to alterations in the prenatal hormonal milieu. The slightest disruption in hormonal activity may cause profound and permanent changes in morphology, physiology and behavior. These changes may result from the interplay of natural maternal hormone production, endocrine disrupting agents delivered through maternal circulation, and/or hormonal transfer from adjacent fetuses of the opposite sex. Some changes, such as ambiguous genitalia, may be visible at birth, while other changes, such as developmental effects on the brain, may not be apparent until puberty or adulthood.

An intrauterine environment that results in low birthweight infants may lead to adult onset diseases such as diabetes and hypertension, according to new research. Since both diabetes and hypertension increase the risk of end stage renal failure, this finding could have major economic implications for health care, highlighting the need to improve prenatal care.

THE INTRAUTERINE POSITION EFFECT

John G. Vandenberg, Ph.D.

Prenatal exposure to sex steroid hormones organizes the brain in ways that affect adult behavior as well as certain anatomical and physiological traits. In many species, males are normally exposed to elevated levels of testosterone in the womb and females are not, except in litter-bearing animals. Prenatal testosterone exposure of the female occurs naturally in litter-bearing animals through hormonal transfer across fetal membranes. Males are similarly affected by the estrogen produced by their sisters. John G. Vandenberg of North Carolina State University,

described this “wombmate” phenomenon, more formally known as the intrauterine position effect, and acknowledged the pioneering work of Frederick vom Saal of the University of Missouri.

A study of female mice exposed to testosterone from adjacent males showed that proximity to male siblings produced anatomical and behavioral variations. Females were classified using vom Saal’s code: developing between two males (2M); with a male on one side (1M); or between two females (0M). Ano-genital distance was longer in 2M females than in 0M females but shorter than in males. The 2M females were leaner than 0M females, with 6 to 7 percent body fat, whereas 0M females had 15-16 percent body fat. The sexually dimorphic nucleus of the preoptic area of the hypothalamus was larger in 2M females than in 0M females, but smaller than in the normal male.

Reproductive characteristics were masculinized in 2M females, including later onset of puberty than 0M females and irregular ovarian cycles. When pregnancy did occur, 2M females produced more males (about 60 percent versus 40 percent in 0M females and 50 percent in 1M females).

Vandenberg and his colleagues are continuing to explore the mechanism underlying this sex-ratio alteration.

Sexual behavior in the 2M females also differed from the 0M females. Males were less attracted to the 2M females than to the 0M females. The 2M females exhibited increased mounting of other females, and displayed less robust lordosis, the typical female mating posture. The 2M females were also more aggressive, adventurous, and more likely to defend food sites. In almost all cases, the 1M females were intermediate between the characteristics of the 0M and the 2M females.

Vandenberg also reported on a new study done with Andrew Hotchkiss, a predoctoral fellow at

the U.S. Environmental Protection Agency, and others that examined how perinatal exposure to environmental anti-androgens affected social play behavior in rats. “Play is directly organized by androgen,” Vandenberg explained, “and male rats play much more frequently—with more rough and tumble—than female rats.” Male rat pups were injected on postnatal day two and three with one of two androgen blocking agents, vinclozolin or flutamide. At the same time, female rat pups were injected with the androgen, testosterone propionate. The pups were raised in same-sex sibling pairs for 35 days, separated for 24 hours, then brought back together in their home cage. Instead of the typical male greeting behaviors and rough-and-tumble play, the males injected with the androgen blockers played more like typical females. The females injected with testosterone showed more male-type play behavior, less rough and tumble than the control group of males, but significantly greater than the control females. This research and similar studies suggest the need to explore other characteristics potentially influenced by endocrine disruptors in the external environment that find their way into the intrauterine environment.

Whether Vandenberg’s results can be extrapolated to humans is unclear, though he cited studies of human fraternal twins suggesting that girls whose twin is a male fetus show greater risk-taking behavior. Research in this area is limited however, given the relative rarity of mixed-sex twins.

EFFECTS OF FETAL EXPOSURE TO NATURAL AND EXOGENOUS STEROIDS

Frederick S. vom Saal, Ph.D.

Shifting the focus from testosterone’s effects on the developing female to estrogen’s influence on the developing male, vom Saal pointed out that there is a wide range for estradiol levels in human fetuses of each sex. Variability factors include

maternal age, first versus subsequent pregnancies, singleton versus multiple birth, race, diet, and geographic location. In mice, for example, vom Saal’s early work showed that males developing between two females (2F males) experience higher biologically active estradiol levels, and, as a result, have larger prostates but smaller seminal vesicles relative to 0F males. The molecular mechanisms mediating those effects are different. In the prostate, estrogen binds to the estrogen receptor (ER), which up-regulates the androgen receptor (AR) gene, increasing AR mRNA and subsequent protein production. The elevated AR expression leads to a lifetime of increased sensitivity of the organism to androgens. In seminal vesicles, however, estrogen modulates 5 alpha-reductase, the enzyme responsible for converting testosterone to dihydrotestosterone (DHT), but not the AR gene. In seminal vesicles, as estrogen binding increases, it down-regulates the enzyme 5 alpha-reductase, diminishing the size of the seminal vesicles.

Estradiol is only one of many estrogens that can invade the intrauterine environment. Estrogenic chemicals in the maternal diet and estrogenic drugs can also disrupt the hormonal milieu, thereby interfering with normal development. vom Saal focused much of his presentation on the effects of these exogenous estrogens and described their additive interaction with background levels of estradiol. As vom Saal explained, it is the biologically active amount of estrogen that matters most: “the free fraction of the steroid, not the bound fraction or the total amount in blood (which includes conjugated estrogen) . . . [is what] is biologically active and clinically relevant.”

If a woman becomes pregnant while using oral contraceptives (an event that occurs an estimated million and a half times each year in the U.S. and Europe), her fetus is exposed to 17-alpha-ethinyl

estradiol. vom Saal cited research in male mice showing that ethinyl estradiol at one-fifth the equivalent dose of oral contraceptives can have the same effects on the reproductive system of the fetus as diethylstilbestrol (DES). The prostate is enlarged, the urethra and seminal vesicles are abnormally small, and daily sperm production is reduced. “What is important about these conditions is there are no external malformations that are visible,” vom Saal emphasized. “You have internal damage without an external indication that the damage is occurring.”

Another synthetic chemical that has estrogenic effects on development is bisphenol A (BPA), an unstable polymer used in the manufacture of polycarbonate plastic. BPA is used to line food cans and is also in dental sealants, baby bottles and many other products. The unstable and lipophilic nature of BPA causes it to leach into food or infant formula. Concentrations equivalent to those experienced by human babies have shown major effects on mouse fetuses. Detectable levels of BPA have been found in human umbilical cord blood, though it is unclear what effects this may have.

The bioactive fraction of BPA that can move into cells is quite large because it does not bind to sex hormone binding globulin, the estrogen binding protein in human plasma, or to alpha fetoprotein in rodents. In some tissues, BPA has the same effect as estradiol, but the effects differ in males and females. Research on male snails, fish, frogs, reptiles, rats and mice has shown that BPA reduces daily sperm production. A 1999 study by vom Saal, Vandenberg and others showed that female mice exposed to BPA during fetal development were heavier than control animals and reached puberty earlier.

Noting that the effects of BPA and other synthetic endocrine disrupting chemicals are visible only

through animal dissection, vom Saal concluded by challenging participants to consider the implications of these effects in humans. Emerging trends in human populations, such as increased incidence of hypospadias and prostate disease, declining sperm counts, epidemic obesity, and accelerated onset of puberty correlate with findings that endocrine disruptors affect animal development. Cause and effect cannot be proven, however. vom Saal concluded his presentation on a cautionary note: “We are told chemicals in the environment are safe, yet for most chemicals health effects studies have not been conducted... We should not conclude that there are no adverse effects until we actually...do the research that is needed. So part of what we are all here about is to distinguish what we know from what we think we know.”

FETAL STRESS RESPONSES: PRENATAL STRESS AND SEXUAL DIFFERENTIATION

Vivette Glover, Ph.D.

Maternal stress during pregnancy can alter the hormonal milieu and markedly affect the developing brain and offspring behavior, as Vivette Glover of the Imperial College, London, UK, explained. Glover cited the early work of Ingeborg Ward, of Villanova University, and colleagues showing that female rats stressed during pregnancy gave birth to male offspring with reduced masculine-type behavior, increased feminine-type sexual and parenting behaviors, and a smaller sexually dimorphic nucleus of the preoptic area. The female offspring showed reduced maternal behavior.

Ward hypothesized these effects as related to fetal/neonatal programming of the hypothalamus-pituitary-adrenal (HPA) axis: the hypothalamus releases corticotropin-releasing hormone (CRH), which acts on the pituitary, causing the adrenals to release cortisol. The cortisol release blocks the normal surge in testosterone that occurs in male fetuses between days 17 and 18 post-conception.

By altering the programming of the HPA axis, which is the main stress response, vulnerability to later behavior problems may be increased.

Glover cited primate studies by Mary Schneider and colleagues at the University of Wisconsin-Madison that validated Ward's findings in rodents. "If a mother monkey is stressed while pregnant, the offspring have a lower attention span, more anxiety, a lot of behavior problems, and you can reproduce this by injecting adrenocorticotrophic hormone (ACTH) into the pregnant mother," Glover explained.

Despite very convincing animal evidence on the fetal effects of maternal stress during pregnancy, there is little research on these effects in humans. Glover reported on work she and her colleagues have done at a London maternity hospital where fetal blood samples are obtained for karyotyping prior to intrauterine transfusion. As she explained, the samples are obtained between 16 and 36 weeks gestation, either "through placental cord insertion, or sometimes through the tummy, which involves piercing the tummy and is stressful for the fetus...so we're in a position to measure the stress responses in the human fetus." Blood samples obtained through the placental cord show no fetal stress response and therefore are used as controls. Examination of samples taken through the uterine wall showed no fetal cortisol response prior to 20 weeks gestation, but a convincing one thereafter.

Fetal basal cortisol levels were highly correlated to maternal cortisol levels, suggesting a possible mechanism whereby maternal stress could affect fetal development. Interestingly, there was also a high degree of correlation between testosterone and cortisol levels in both male and female fetuses, which could mean that high cortisol levels lead to masculinization of female fetuses. In another study, Glover's colleague Jeronima Teixeira of the

Imperial College, London scanned blood flow through uterine arteries of pregnant women, using color Doppler ultrasound. The women's anxiety was assessed using a self-rating questionnaire. Investigators found that mothers who were anxious had significantly reduced blood flow to the fetus, heightening the risk of intrauterine growth restriction.

To study long-term effects of maternal stress during pregnancy, a longitudinal prospective study was initiated in 1991 in Avon, England. The study enrolled more than 7,000 women and their offspring. Maternal anxiety was assessed at 18 and 32 weeks antenatally and at 8 weeks, 21 and 32 months postnatally. At 47 months postnatally, the mothers completed a questionnaire assessing several dimensions of their child's behavior, including hyperactivity/inattention, emotional problems (anxiety and depression), and conduct disorders. Investigators found that maternal anxiety at 32 weeks gestation, when corticoid receptors are developing, was a significant risk factor for behavioral problems in both boys and girls. Sons of anxious mothers had twice the risk for hyperactivity and attention disorders, compared with control boys. This effect was not apparent in the girls studied.

This longitudinal study also showed that prenatal maternal stress affected laterality (handedness), with offspring of women who were anxious at 18 weeks gestation more likely to be mixed-handed than those whose mothers were anxious at 32 weeks gestation or 8 weeks postpartum.

However, there were no sex differences in handedness related to prenatal anxiety. "It's interesting," Glover said, "that atypical handedness has been associated with altered sexual orientation, autism, dyslexia, attention deficit/hyperactivity, and schizophrenia. My hypothesis is that it's possible that all these things are actually partially caused by prenatal stress and anxiety. Clearly all

of them are multifactorial or have a genetic component, but it looks as though this group may be exacerbated by the emotional state of the mother during pregnancy.”

Glover and her colleagues are now studying the effects of maternal and fetal stress during birth and immediately postpartum. Elective cesarean delivery, emergency cesarean, normal delivery and assisted delivery are equally stressful for the mother but have different effects on the fetus. Early results (up to eight weeks) show that assisted delivery is the most stressful and elective cesarean is the least stressful for the fetus.

FETAL ORIGINS OF ADULT DISEASES: A HYPOTHESIS

Daniel T. Lackland, Dr.P.H.

The quality of life in the womb has lasting effects on health. Citing David Barker of the Medical Research Council at Southampton University, England, who works on fetal origins of adult onset diseases, Daniel T. Lackland of the Medical University of South Carolina, presented data on how low birthweight in South Carolina is associated with stroke, end-stage renal disease, early adult onset hypertension, and diabetes, all of which have major public health implications.

People born in South Carolina have the lowest life expectancy in the United States. The state is located in the center of the southeastern “stroke belt,” recognized since 1950 as an area with unusually high death rates from stroke. Lackland and his colleagues compared stroke incidence rates in South Carolina with incidence rates from stroke in Minnesota, Missouri, Connecticut, Massachusetts, and the Netherlands, and found that the incidence of stroke was highest for South Carolina in every age cohort. In addition, people from South Carolina have a 10-year earlier onset of disease. For example, a person who is between

the ages of 55 and 64 years old in South Carolina has a cerebrovascular health status equivalent to someone in Minnesota between the ages of 65 and 74. “In addition to the geographic disparity in disease, racial differences in stroke are even more obvious,” Lackland emphasized. “African-Americans have two times greater risk of stroke than Caucasians, with a 10-year differential in age of onset - 10 years earlier than Caucasians. Thus, an African-American female in South Carolina has the stroke risk of a female in Minnesota who is 20 years older.”

The geographic and racial disparity in disease risks is also evident with stroke subtypes. Incidence of hemorrhagic stroke, a more severe type of stroke, is dramatically elevated in African-Americans in South Carolina. At particular risk are African-Americans under 55 years of age. About 50 percent of the strokes that occur in African-Americans are hemorrhagic, whereas the expected proportion would be about 10-15 percent.

Geographic and racial disparities are also apparent in end-stage renal disease in South Carolina. “South Carolina has the highest prevalence and incidence of end-stage renal disease in the entire country,” Lackland explained. “While a third of the general population in South Carolina is African-American, two-thirds of the population on dialysis is African-American.” In addition to the health burden, end-stage renal disease is costly. The direct costs are approximately \$72,000 a year to maintain an individual on dialysis.

The major risk factors for stroke and end-stage renal disease are diabetes and hypertension, both of which are more prevalent among African-Americans than whites in South Carolina. African-Americans have nearly a two-fold greater prevalence of Type 2 diabetes and have higher levels of glycohemoglobin (HgbA1c) than whites. Likewise, nearly 37 percent of African-American

men in South Carolina are hypertensive compared with 29 percent of white men in the state. Nearly 40 percent of African-American women in South Carolina are hypertensive compared with 23 percent of white women in South Carolina.

Compliance with hypertensive therapy is slightly better among women than men but nearly 60 percent of South Carolina hypertensive women and more than 87 percent of hypertensive African-American men have uncontrolled hypertension.

Viewing the geographic, racial and gender disparities in these chronic conditions through the lens of the Barker hypothesis, Lackland and his colleagues saw another disparity: low birthweight. "African-Americans have nearly a twofold greater risk of being low birthweight than do their white counterparts," Lackland noted, "And residents of the Southeast have an even greater burden, with higher rates of low birthweight babies than other regions of the country."

The "Fetal Origins of Adult-Onset Disease" hypothesis was tested using birth certificate data and the data on the Medicaid population in South Carolina born in 1950 and later. Using the Medicaid database, effective controls for socioeconomic status are maintained. Controls were matched for age, race, and sex. Researchers found a consistent correlation between low birthweight and hypertension in both blacks and whites and males and females. A similar pattern was apparent for diabetes, stroke, and congestive heart failure, with the smallest babies having the greatest risk of these chronic health problems.

Lackland reported on another study, which compared birthweights of patients under age 50 who had end-stage renal disease (ESRD) to birthweights of non-ESRD controls matched for age, sex and race. Low birthweight was consistently associated with ESRD in both men and women as well as blacks and whites, whether the ESRD

resulted from diabetes, hypertension or other causes.

Although the association between low birthweight and hypertension remains unexplained, some clues are emerging from Lackland's work and from Barker's studies in England. Age-related increases in systolic blood pressure are related to stiffening of the vascular endothelium. Early onset of vascular stiffening in adults with a history of low birthweight may be due to a lifetime of insufficient elastin, the major connective tissue protein in arteries and blood vessels.

Lackland also noted important race and sex differences in responsiveness to anti-hypertensive medications. For example, among those with a history of low birthweight, white males tended to be more responsive to ACE inhibitors whereas African-American women seemed to respond better to a calcium channel blocker.

Low birthweight is primarily due to prematurity, which has developmental implications for specific organs, particularly the kidney. In humans, more than half the normal complement of nephrons is developed during the third trimester. Babies born at 30 weeks gestation have fewer nephrons than those born at 36 weeks, which may lead to impaired renal function. As Lackland explained, "Suppose you're born with a lower number of nephrons. Suppose you're diabetic, suppose you're hypertensive and you're putting continual stress on those kidneys... Maybe the number of nephrons does play a role, maybe an active role." Impaired renal development may also affect response to angiotensin II blockers.

Lackland also cited the work of his graduate student Pam Ferguson who is using similar methodology to study breast cancer among women under 50 in relation to birthweight. Ferguson has found that high birthweight may increase the risk of

early breast cancer.

In response to a participant's question about inter-generational effects, Lackland noted that birthweight is only one factor in the fetal origins of adult onset disease. "If it's just low birthweight, then everybody in India should be hypertensive and diabetic because the average birthweight in India is 2700 grams," Lackland said. "But in India, a low birthweight female baby stays slim and becomes a lean adult woman with little or no diabetes and no signs of hypertension, a very healthy individual. However, a different scenario is seen for males. There seems to be a tendency for young males to be given a high calorie diet, resulting in increased body mass and obesity in adolescence. We think that combination of being low birthweight and then being obese at an early age tremendously precipitates the increased risk of diabetes and hypertension, and subsequent stroke and end-stage renal disease."

CONGENITAL DISEASES

Congenital diseases and disorders were defined broadly in this session to include genetic diseases and conditions that are transmitted from parent to child, such as Trisomy 21 and Duchenne muscular dystrophy, and single birth defects such as congenital hip dysplasia and spina bifida. Clearly, sex matters in congenital diseases and conditions—their occurrence, nature, and/or transmission—not only the sex and the sex chromosomes of the affected individual but also the sex of the parent who contributes a given allele. In addition, congenital disorders such as congenital adrenal hyperplasia may affect behavior.

SEX DIFFERENCES IN MENTAL RETARDATION

Charles J. Epstein, M.D.

Genomic technologies are beginning to explain why males constitute a majority of the mentally retarded population ($IQ \leq 70$). As Charles J. Epstein of the University of California, San Francisco, explained, many disorders that cause mental retardation occur more frequently and/or more severely in males because the disorders are X-linked.

About 1 in every 500 males will have some form of X-linked mental retardation, accounting for between 13 and 21 percent of the mentally retarded male population. The forms of X-linked mental retardation fall into two categories: non-specific X-linked mental retardation and syndromic forms of X-linked mental retardation. Based on family studies, an estimated 20 to 25 genes on the X chromosome are thought to contribute to non-specific types of mental retardation. “These are being individually mapped and eventually we should know what these genes are,”

Epstein said.

In syndromic X-linked mental retardation, the retardation is only one of a broader constellation of characteristics. Epstein used the example of fragile X syndrome, which occurs approximately twice as often in males as in females, and is characterized by a long face with a large jaw, large skull and prominent ears. Postpubertal males develop enlarged testicles and some elements of a connective tissue disorder.

Fragile X syndrome is caused by triplet repeat expansion of a CGG sequence in the *fmr-1* gene on the X chromosome. Normal individuals have between 6 and 50 copies of the CGG repeat, whereas individuals with fragile X syndrome have 230 to 1000 copies. A “normal transmitting male,” who has an intermediate number of repeats (50-230), can transmit this “premutation” to his daughter. The triplet repeat is likely to expand in future generations. If the expansion occurs in the daughter’s gametes, any of her sons would have a 50% chance of being affected.

Epstein pointed out that X-inactivation can also affect expression of disorders such as fragile X syndrome if a mutant allele is transmitted to a female. If one X is preferentially inactivated in a majority of cells during the normal process of X-inactivation in development—X-inactivation skewing—a better or worse phenotype can result. The outcome depends on whether the skewing is toward the normal or the mutant allele-harboring X chromosome.

X-linked mental retardation also occurs in females but less frequently than in males. Epstein described Rett syndrome, a leading cause of mental retardation in females. Girls with this disorder appear normal at birth and develop normally until sometime between 6 and 18 months of age, at which time regression begins. Head growth slows and any expressive language skills acquired are

lost. Motor function deteriorates, leading to repetitive hand movements, shakiness of torso and limbs, and ataxic gait. Seizures and breathing problems are common.

Until genomic technologies revealed the cause of Rett syndrome, it was often misdiagnosed as cerebral palsy, autism, or non-specific developmental delay. Epstein cited the work of Huda Zoghbi of Baylor College of Medicine, and Uta Francke of Stanford University and their colleagues who identified the mutations on the X-linked *mecp2* gene responsible for Rett syndrome. *Mecp2* normally produces a transcriptional repressor protein, MeCP2, which helps regulate neural development. It is hypothesized that mutations in *mecp2* limit production of the repressor protein.

Once *mecp2* was cloned, researchers discovered hundreds of mutations, most of them deletions, associated with a wide spectrum of symptoms ranging from classical Rett syndrome to mild neurological and learning impairments to no symptoms at all. Epstein explained that a lack of symptoms may be related to mutations in non-essential regions of the gene and/or X-inactivation skewing toward the normal X chromosome in females.

Although Rett syndrome was long believed to be lethal in males, DNA analysis has proven otherwise, showing that non-lethal *mecp2* mutations exist in males with a variety of neurological defects. Recent research implicates this gene in other neurodevelopmental disorders, including autism.

Using autism as an example, Epstein also addressed complex disorders—conditions involving more than one abnormal gene. “There is a lot of evidence that autism has a genetic etiology,” he explained. “Recurrence risk in families is signifi-

cant—on the order of 3 to 5 percent. Although the incidence of autism is quite low in the population, if there are two affected kids in the family, the risk for the next one is 25 percent.” He also pointed to the high concordance rate for autism in monozygotic twins, which is an active area of current research. Another area of intense investigation is why autism affects four times more males than females. “There are hypotheses that have to do with combinations of imprinted genes on the X chromosome and X-inactivation; whether those are true or not, we do not know,” Epstein said.

Mental retardation may also be related to abnormalities in the number of sex chromosomes (aneuploidy), which is also more common among males than females (1/400 males versus 1/650 females). Epstein cited the seminal work of Arthur Robinson of the University of Colorado whose early prospective study has followed individuals with sex chromosome aneuploidy since birth. Types of sex chromosome aneuploidy include XXY, XXX, XYY, and 45,X. Each affect cognitive development in various ways. Epstein noted that in XXY individuals, verbal IQ is about 10 points lower than controls and full score IQ is about 5 points lower than controls. Cognitive development is more impaired in XXX individuals, particularly in verbal skills. They exhibit lower verbal and performance IQ and full scale IQ may be a full 18 points below controls. In XYY individuals, however, no significant differences in cognitive function are apparent.

The aneuploidy of greatest research interest is 45,X, or Turner syndrome. Although females with Turner syndrome are usually not mentally retarded, recent reports indicate that those who receive a paternal X chromosome have higher verbal IQ scores than those who receive a maternal X chromosome. This finding has generated controversy and requires further study.

The sex of the parent contributing a given chromosome also influences the occurrence and transmission of chromosome abnormalities that give rise to mental retardation. One example of this effect is Trisomy 21, caused by a parent with a Robertsonian translocation, in which chromosomes 14 and 21 are stuck together. Although a parent who carries a balanced translocation (i.e., the full complement of both chromosomes is present in the individual) is unaffected by the abnormality, he or she can transmit the translocation in an unbalanced form. As Epstein explained, "If the father is the carrier. . . the probability of having a child with Down syndrome would be about one and a half percent, but if the mother is the carrier, the probability would be about 15 percent." This difference is likely due to female meiosis being relatively tolerant of translocations, whereas male meiosis is not very tolerant, often leading to male infertility. For the vast proportion of Trisomy 21 not associated with a translocation, the probability of giving birth to a child with Down syndrome depends on maternal age; an exponential increase begins between 30 and 35, and by age 45, the likelihood of having a child with Down syndrome is two to three percent.

Another example of mental retardation related to the sex of the parent contributing the abnormality is the prototype deletion in the q11-q13 region of chromosome 15, which results in Prader-Willi syndrome (PWS) if the deletion affects the paternally derived chromosome, or in Angelman syndrome (AS) if it affects the maternally derived chromosome. Although PWS and AS are caused by deletions in the same chromosomal region, they exhibit totally distinct phenotypes, exemplifying the essential parent of origin effects. Mental retardation is a facet of both syndromes, but it is more severe in AS than in PWS.

FEMALES ARE UNIQUE MOSAICS: THE PATH FROM MUSCULAR DYSTROPHY TO RECURRENT PREGNANCY LOSS

Cheryl A. G. Scacheri, M.S.

Duchenne muscular dystrophy (DMD) is the most common type of muscular dystrophy, a progressive degenerative disease. Like most X-linked diseases, DMD occurs primarily in males (1/3500 live births). As Cheryl A. G. Scacheri formerly of the Children's National Medical Center in Washington, DC (now at GeneDX), explained, "Males who have a mutation in the dystrophin gene have full-blown Duchenne muscular dystrophy. Females with a copy of the mutated dystrophin gene are carriers of this form of muscular dystrophy." However, skewed X-inactivation can drive the expression of this disorder in females, who are called "manifesting carriers".

The dystrophin gene is located at Xp21 on the short arm of the X chromosome. This very large gene (2.3 million base pairs) is subject to many mutations; about 60 percent of mutations found are large deletions and about 5 percent are large duplications. Boys with DMD appear normal at birth and develop motor skills on schedule until about age 4 when they begin to have trouble climbing stairs and rising from a sitting position. The disorder is often diagnosed by age 5 when the inability to keep up with peers becomes readily apparent. Much of the weakness is in the pelvic girdle and the shoulder girdle. Between ages 10 and 15, most boys with DMD will lose the ability to walk. Wasting also includes the heart muscle and the diaphragm, which usually causes death before age 30.

About two-thirds of male cases result from maternal transmission of a dystrophin gene mutation; one-third are attributable to new spontaneous mutations. In most manifesting female carriers of

DMD the mutation is a de novo event on the paternal dystrophin gene, and skewing of X-inactivation must occur for these women to exhibit the disease.

Males with DMD completely lack dystrophin protein in their muscle fibers, whereas female carriers have a mosaic pattern of protein expression in their muscle. This mosaic pattern consists of some fibers that produce normal dystrophin and others completely lacking the protein because the normal dystrophin gene is inactivated and the mutated active gene cannot produce the normal dystrophin protein. The cells positive for the dystrophin protein are those in which the normal gene resides on the active X chromosome. "If more than 25% of [normal levels of] dystrophin is produced, the individual is protected from having symptoms," Scacheri explained. Female carriers with mild muscle weakness have between 15 and 25% of normal dystrophin levels. The rare females who do have severe DMD are clinically indistinguishable from males with DMD. "They have extremely skewed X-inactivation," Scacheri said, "so nearly all of their cells are using the X chromosome that does not produce dystrophin."

Scacheri reminded participants that X-inactivation skewing has several possible causes, including (1) chance, which causes mild to moderate skewing, (2) monozygotic twinning, in which X-inactivation begins before the embryo divides and may unevenly distribute cells with a particular inactive X between the embryos, (3) mutations or polymorphisms in genes that regulate X-inactivation, (4) any event that offers a proliferative advantage to cells with a particular X active, such as cancer, and (5) chromosome defects, such as translocations or deletions. Scacheri and her colleagues are also investigating single gene changes to determine whether they might also be involved in skewed X-inactivation.

A rare case of DMD in a two-year-old girl afforded Scacheri's colleague, Elena Pegoraro, and others in Eric Hoffman's laboratory at George Washington University, the opportunity to study one of the first families to show *inherited* extremely skewed X-inactivation. The child's muscle biopsy showed abnormal dystrophin staining, and X-inactivation studies revealed that the same X chromosome was active in virtually all of her cells—with skewing of more than 95 percent. Her mother and grandmother and many other female family members also were shown to have extreme skewing, preferentially using the normal paternally-transmitted X chromosome.

Further analysis of the pedigree showed that women who had highly skewed X-inactivation had more daughters than sons, whereas family members who did not show skewing had equivalent numbers of boys and girls. The analysis also showed that the women with highly skewed X-inactivation had more than twice the rate of spontaneous abortion compared to female relatives (32 percent versus 15 percent, the rate seen in the general population).

To follow up on these findings, Mark Lanasa of the University of Pittsburgh School of Medicine, initiated a case-control study that enrolled 100 women with two or more first-trimester pregnancy losses. He found that 14 percent of these women showed greater than 90 percent skewed X-inactivation. In the control population, which comprised 100 women who had one successful pregnancy and no pregnancy losses, only one woman showed greater than 90% skewed X-inactivation. Scacheri reported that other groups, including Wendy Robinson of the University of British Columbia, and S. Uehara of Tohoku University, School of Medicine, Sendai, Japan, have found similar results: between 14 and 18 percent of women with recurrent spontaneous

abortion show highly skewed X-inactivation. “There are differing opinions on what the explanation might be,” Scacheri noted. She and her colleagues are investigating whether mutations in X-linked genes critical to development might explain why these women’s cells exhibit skewing – presumably away from the mutation, whereas Robinson’s group is studying a possible chromosomal etiology. “We have two families with second trimester male pregnancy losses,” Scacheri reported. “The interesting thing is that in blood we’re finding skewed X-inactivation but in oral cells we’re finding random X-inactivation. If we can narrow down the region of the X chromosome where the [presumed] mutant genes reside, then we can use this knowledge to make a reasonable candidate gene approach to determining what the genetic problem might be.”

CONGENITAL ADRENAL HYPERPLASIA: PRENATAL ANDROGENS AND BEHAVIORAL SEX DIFFERENCES
Sheri A. Berenbaum, Ph.D.

More than two decades of research have changed the question about the effects of prenatal androgen exposure on physical and behavioral sexual differentiation. As Sheri A. Berenbaum of the Pennsylvania State University, noted, researchers no longer ask *whether* this exposure causes powerful and permanent effects in humans but *how?* Berenbaum and her colleagues have explored this question for more than 15 years, using the model of congenital adrenal hyperplasia (CAH), an autosomal recessive disorder occurring in about 1 out of 10,000-15,000 live births.

CAH is caused by an enzyme defect in 21-hydroxylase, which results in an inability to synthesize cortisol, and thereby exposes the fetus to elevated levels of adrenal androgens throughout gestation. Girls with CAH are born with masculinized genitalia. However, as the work of Berenbaum and others on girls with CAH and their siblings has

shown, masculinized behavioral changes persist in girls with CAH even after surgery in the neonatal period to convert CAH.

Using objective multiple measures of behavior, Berenbaum and her colleagues focused on behaviors that show sex differences, including observed toy play, activities and interest, playmate preference, interest in infants, and gender identity. The study began with a small group of children who were then followed over 15 years even as other children were added into the study. Behaviors were measured four times between ages 3 and 18. Children in the original group are now in late adolescence and early adulthood so the investigators are beginning to look at sexual behavior as well.

Berenbaum reported that girls with CAH play more with boy’s toys (transportation and building toys) than their sisters do. When offered a toy to take home, girls with CAH are also more likely to choose a transportation toy. They also play less with girl’s toys than their sisters. However, there was no observable difference in the toy play behavior of boys with CAH and their unaffected brothers.

Studied in adolescence (through self-report and parent-report), this cohort of girls with CAH displayed interests and activities very different from their sisters’. There was no overlap in interests and activities between the control boys and the control girls. Girls with CAH were in between the control boys and the control girls.

It has been hypothesized that the masculinized behaviors of girls with CAH are related to parents’ treatment of the girls because of masculinized genitalia. However, as Berenbaum pointed out, data from Swedish researchers have shown that boy-typical behavior is directly related to the genotype and not to parental treatment. Girls

with the null CAH mutation have no 21-hydroxylase (21-OH) enzyme activity and therefore the greatest degree of prenatal androgen excess. These girls spent the most time playing with boy's toys, even with parents present. Girls with a milder form of CAH have some 21-OH activity and therefore lower levels of prenatal androgen excess. These girls spent less time playing with boy's toys, whether parents were present or not. Berenbaum has initiated a prospective study of girls with CAH detected through newborn screening to explore these effects further.

Although girls with CAH showed both physical and behavioral differences from their sisters, their gender identity was typically female. Using an instrument with questions such as "Are you happy being a girl?" and "Do you ever wish you could be a boy?", the responses from girls with CAH and their sisters were similar. In response to the question, "If there was a magical way to turn you into a boy forever, would you do it?" neither the girls with CAH nor the control group was interested.

Compared with their sisters, girls with CAH are much less interested in babies and have higher spatial ability. "The androgen effect on the sex difference in spatial ability is much larger than it is on sexual orientation," Berenbaum explained. "The difference between CAH females and controls is relatively small in terms of the sex of the partner they have sexual arousal to."

Citing the work of Sally and Bennett Shaywitz and colleagues at Yale University on sex differences in brain activity, Berenbaum showed a functional MRI (fMRI) of male and female brain activation in response to a rhyming task. The slide revealed that the male used the left hemisphere but the female used both the left and right hemispheres. Berenbaum and her colleagues are beginning to study MRIs of the original cohort of

CAH girls as they get older to identify any sexual dimorphisms in brain activity and how they affect performance function, particularly mathematical and mechanical abilities.

SEX AND THE DEVELOPING BRAIN

Like the genitalia, the brain begins as a bipotential organ, equally capable of assuming a male or female phenotype. Hormone action during development determines the outcome by controlling the numbers of neurons in the various parts of the brain and establishing the connections between them. The developing mammal is exposed not only to hormones of fetal origin but also to maternal hormones of pregnancy and lactation. Many of the neural effects of hormones are cell-type- and temporally specific. In the final session of the conference, speakers explored the mechanisms by which hormone action on the brain is mediated by a variety of signaling pathways.

CELLULAR MECHANISMS UNDERLYING DEVELOPMENT OF SEXUALLY DIMORPHIC NEURAL PATHWAYS

Richard B. Simerly, Ph.D.

During the past 15 years, research has revealed an entire circuitry in the mammalian forebrain that is sexually dimorphic. As Richard B. Simerly of the Oregon Health Science University, explained, in most of the sexually dimorphic regions of the rodent brain, males have more neurons than females, but in a few of the regions, females have more neurons. The latter is true for the anteroventral periventricular nucleus (AVPV).

Located at the rostral end of the hypothalamus, the AVPV controls ovulation. Most of the neurons in the AVPV abundantly express estrogen and progesterone receptors, making them responsive to circulating levels of these hormones. The AVPV connects to gonadotrophin-releasing hormone (GnRH) producing neurons, which direct gonadotrophin secretion. The *output* pathway

from the AVPV is more robust in females than in males, however, the sensory *input* pathways into the AVPV tend to be more robust in males than in females.

Simerly emphasized that hormones can promote or inhibit cell survival, or can influence the number of cells in brain regions in cell-type-specific ways. In the AVPV, for example, there is more neuron production of the opioid peptide proenkephalin in males than in females. However, early postnatal exposure to testosterone will initiate a complete sex reversal in the characteristics of these neurons.

Simerly presented several mechanisms of estrogen signaling in the brain. These include indirect genomic effects that are conveyed to the nucleus through a variety of second messengers and transcription factors. However, the best characterized pathway for estrogen signaling is the classic direct genomic mechanism through which the hormone enters the cell and binds to a nuclear receptor. The receptor acts as a transcription factor to alter the expression of a variety of genes that affect the cell, including influencing cell survival, Simerly said.

Simerly and his colleagues demonstrated the developmental action of the estrogen receptor alpha ($ER\alpha$) by studying mice in which the receptor had been deleted. The $ER\alpha$ deletion blocked the sexual differentiation of a population of dopamine neurons. This same receptor appears to be essential to the formation of a sexually dimorphic neural pathway that delivers pheromone information to the AVPV. Reporting on a series of *in vitro* experiments, Simerly noted that development of this pathway required hormone exposure of the AVPV and the presence of $ER\alpha$. $ER\alpha$ directly influences the number of cells that mature in specific brain structures, and specifies the formation of neural pathways between these

cell groups. Thus, ER α plays a crucial role in determining the sexually dimorphic architecture of forebrain pathways.

MECHANISMS OF STEROID MEDIATED BRAIN DIFFERENTIATION

Margaret M. McCarthy, Ph.D.

The rat brain is useful for studying cellular mechanisms of hormone action because it has two functional sexual dimorphisms: control of reproductive behavior, and control of gonadotrophin secretion. As Margaret M. McCarthy of the University of Maryland School of Medicine, pointed out, a true dimorphism exists in male and female rats. In contrast, sex differences in the human brain are allomorphic, i.e., different but not genuinely opposite.

Sexual dimorphisms in the rat are also hormonally manipulatable and reversible. Exposure of the female to testosterone during the sensitive prenatal period results in male-type behavior in adulthood. Castration of the male at birth and exposure to estrogen in adulthood lead to female-type behavior.

McCarthy noted that different brain regions control different functions, citing as examples the medial preoptic area (mPOA) as a critical region for control and expression of male sexual behavior, the ventral medial nucleus (VMN) as a major site controlling female sexual behavior, and the arcuate nucleus as a modulator of the luteinizing hormone surge. "None of these areas work in isolation," McCarthy said, "yet they are the major components of the system...places to look for sexual dimorphisms and try to get at mechanisms."

McCarthy raised three issues for participants to consider: (1) some mechanisms are unique to a single brain region; (2) some mechanisms are

common to several brain regions; and (3) a mechanism that is advantageous in one brain region could be deleterious in another. Citing Simerly's discussion of the AVPV as an example of mechanisms unique to a single brain region, McCarthy described gamma-aminobutyric acid (GABA) signaling as a mechanism common to several brain regions, but also as a mechanism that could be advantageous in one region but deleterious in another.

GABA is a predominant neurotransmitter, produced only by neurons, to which almost every cell in the brain is sensitive. "GABA has a dual identity," McCarthy explained. "In addition to being an important inhibitory transmitter in the adult brain, we now know that it is actually an excitatory transmitter in the developing brain. GABA is believed to be the primary source of excitation as the brain is developing."

The developmental shift from excitatory to inhibitory GABA action is affected by perinatal hormones and therefore differs in males and females. McCarthy reported that excitatory GABA activates voltage-gated calcium channels indirectly (by depolarizing the membrane), resulting in a transient increase of cytosolic calcium. "Calcium is the elixir of life to a developing neuron," McCarthy said. Calcium is not only essential to the survival of the neuron, but determines its size, how many times the neuron branches and the course of synaptogenesis.

Neurons from the male brain that are exposed to estradiol exhibit calcium transients twice the amplitude of unexposed neurons. Estradiol-exposed neurons are also more likely to respond to GABA with membrane depolarization as development progresses. However, GABA action is not uniform throughout the brain. McCarthy explained, "By late gestation, the hypothalamus is beginning the shift toward hyper-polarizing [of

the cell membrane] in response to GABA. At around birth, when we have a massive increase in testosterone in males, we have a convergence between the shift of de-polarizing to hyper-polarizing GABA action. High steroid levels result in the potential for foci of excitatory GABA in the hypothalamus, where they can act to differentiate the brain toward the male phenotype by increasing these calcium transients. In the female, we have a more uniform shift toward the hyper-polarizing effects of GABA.”

Increased levels of intracellular calcium activate kinases, which stimulate the transcription factor known as CREB (cAMP-response-element-binding protein). McCarthy reported that GABA receptor activation triggered a dramatic induction of CREB activation in some regions of the male brain but a reduction of CREB activation in some regions of the female brain. These distinct patterns of activation and deactivation of CREB emphasize the regional heterogeneity and divergence in excitatory versus inhibitory GABA action that is postulated to orchestrate the development of a male brain versus a female brain.

McCarthy pointed out that GABA’s potential for excitotoxicity could be detrimental in the hippocampus, which is particularly vulnerable to ischemic damage. Premature infants are at high risk of ischemia, which triggers release of glutamate and high levels of GABA. To examine the effects of ischemia on the hippocampus, a GABA agonist was administered to newborn rat pups at birth and on the following day. Assessment of the hippocampus on postnatal day 7 showed reduction in hippocampal volume in both males and females and greater cell death in males. This suggests that GABA may contribute to increased excitation in the male brain, predisposing it to injury or other developmental disorders.

McCarthy noted that this area of research is ripe

for exploration. “We really are at the baby-step stage of figuring out what’s going on in the brains of males and females [during development].”

EFFECTS OF MATERNAL/FETAL INTERACTION ON NEURAL DEVELOPMENT

Christine K. Wagner, Ph.D.

Estradiol and testosterone are not the only hormones influencing the development of sex differences in brain and behavior, according to Christine Wagner of the State University of New York, Albany. Maternal hormones, particularly progesterone, which circulates at high levels during gestation and lactation, may also play a role.

Wagner presented her findings on progesterone effects on the brain of pregnant rats and their developing fetuses. Progesterone levels peak between gestational days 15 and 19, then drop precipitously just prior to birth (day 23). The close correlation of maternal and fetal progesterone levels points to the mother as the source of progesterone in fetal circulation. When Wagner and her colleagues examined progesterone receptor (PR) immunoreactivity in the medial preoptic nucleus (MPN) of male and female rats, they found that males express high levels of PR whereas females express very little. The sex difference in expression of PR extends from gestational day 19 to at least postnatal day 7, but at about postnatal day 10, the female starts to express significant levels of PR, thereby decreasing the magnitude of the sex difference. During this critical developmental window, the male MPN is more sensitive to progesterone than the female MPN.

As Wagner explained, this sex difference in PR expression depends on the differential exposure of males and females to testosterone, which is aromatized to estradiol in the male. “Prenatal exposure of females to either testosterone propionate or DES significantly increased PR immunoreactivity

levels in females to levels comparable to those seen in normal males.” However, dihydrotestosterone did not increase PR levels in females, suggesting that fetal testosterone, which is aromatized to estradiol, induces PR expression in the male MPN, causing the sex difference.

Wagner also reported on a study in which neonatal female rats and castrated male rats were exposed to testosterone and RU-486, a progesterone receptor antagonist, producing sexually differentiated responses. In females, RU-486 attenuated the masculinizing effects of testosterone on the volume of the central subdivision of the MPN (MPNc). In males, however, RU-486 augmented the effects of testosterone. “It seemed like RU-486 made supermales,” Wagner said. These findings suggest that males and females are differentiated at birth in a way that postnatal testosterone cannot counter.

A study of PR immunoreactivity in the ventromedial nucleus (VMN) of the hypothalamus also showed a more subtle but significant sex difference. In this region, females had slightly more PR expression than males, and were unaffected by prenatal testosterone treatment. Wagner emphasized that they still don’t have a good explanation for how this sex difference is being regulated in the VMN. “The rules of sexual differentiation are very complex and they’re not just simply having testosterone or not having testosterone. The sex difference in the VMN may be influenced by maternal and/or placental hormones, or there may be a direct influence of genetic sex. These questions remain unanswered.”

SPEAKER BIOGRAPHIES

Sheri A. Berenbaum, Ph.D.

Dr. Berenbaum is currently Professor of Psychology at Pennsylvania State University. She received her Ph.D. from the University of California, Berkeley, and then completed a postdoctoral fellowship in behavioral genetics at the University of Minnesota. She has held faculty positions at Southern Illinois University School of Medicine and Finch University of Health Sciences / The Chicago Medical School.

Berenbaum is particularly interested in the behavioral effects of prenatal sex hormones. Her behavioral studies of children and adults with congenital adrenal hyperplasia have been supported by the National Institutes of Health since 1985. She served on the Institute of Medicine Committee that wrote the report "*Exploring the Biological Contributions to Human Health: Does Sex Matter?*"

Charles J. Epstein, M.D.

Dr. Epstein was educated at Harvard College and Harvard Medical School and trained in internal medicine at the Peter Bent Brigham Hospital, in medical genetics at the University of Washington, and in biochemistry at the National Institutes of Health. Since 1967, he has been at the University of California, San Francisco, where he is now Professor of Pediatrics, Chief of the Division of Medical Genetics, and Co-Director of the UCSF Program in Human Genetics.

Dr. Epstein's major research interests have been in the biochemistry and genetics of early embryonic development, the pathogenesis of Down syndrome, and genetic approaches to the study of free radical defense mechanisms.

Vivette Glover, Ph.D.

Dr. Glover studied biochemistry at Oxford and obtained her Ph.D. from London University. She then worked in biological psychiatry at Queen Charlotte's and Chelsea Hospital. More recently she has applied this expertise to the problems of mothers and babies and is currently Professor of Perinatal Psychobiology, at the Imperial College, London. She runs the "Fetal and Neonatal Stress Research Group" at the Institute of Development and

Reproductive Biology, a multidisciplinary team, interested in the long term effects of perinatal stress. Recent projects of interest include a study showing that maternal anxiety during pregnancy doubles the risk for hyperactivity in boys; studies showing possible mechanisms by which maternal anxiety may affect the development of the fetus; a study showing that babies born by different methods (elective caesarean, normal vaginal, assisted) have different stress and crying responses at 8 weeks; and the first trial of analgesia in the fetus.

Andrew R. Hoffman, M.D.

Dr. Hoffman is Professor of Medicine and Molecular and Cellular Physiology at Stanford University School of Medicine. He has previously served as Chief of the Medical Service at the VA Palo Alto Health Care System, as Chief of the Division of Endocrinology, Gerontology and Metabolism at Stanford, and as Associate Chair of the Department of Medicine. Dr. Hoffman is an international authority on genomic imprinting and the role of growth hormone and insulin-like growth factors in aging, cellular growth and metabolism.

Randy L. Jirtle, M.S., Ph.D.

Dr. Jirtle completed his undergraduate work and received both an M.S. and Ph.D. in radiation biology from the University of Wisconsin-Madison.

Currently he is the Professor of Radiation Oncology and Associate Professor of Pathology at Duke University. He is also the current Director of the Division of Radiation and Molecular Oncology Research, on the Board of Directors of Duke University's Integrated Toxicology Program, and a member of the Cell and Molecular Biology Training Program.

His research focuses on determining the role of genomic imprinting in carcinogenesis. Of particular interest is the imprinted M6P/IGF2R gene, which functions in tumor suppression.

Daniel T. Lackland, Dr.P.H.

Dr. Lackland is professor in the Department of

Biometry and Epidemiology, and the Division of Cardiology at the Medical University of South Carolina. He received his doctorate degree in cardiovascular epidemiology from the University of Pittsburgh.

Much of his research interest involves the population risk assessment of cardiovascular disease, stroke and hypertension. In particular, his work focuses on the factors associated with the racial disparity in disease and tissue samples from the Charleston Heart Study and Evans County Heart Study. He is currently collaborating with Professor David Barker at the Medical Research Council with a study of the fetal origin of hypertension-related diseases and endothelial function. In addition to these epidemiological investigations, he is involved in population high blood pressure control efforts. He is involved with an assessment of the quality of hypertension treatment and control in the South Carolina Medicaid population. He serves on the boards of the Hypertension Initiative and the Diabetes of South Carolina. He also leases blood pressure screening activities at sporting events in the Southeast, and is a consultant for high blood pressure control programs in Latin America and central Asia.

Margaret M. McCarthy, Ph.D.

Dr. McCarthy completed her undergraduate work and received a master's degree from the University of Missouri in 1984 and a doctoral degree in behavioral and neural sciences from the Institute of Animal Behavior at Rutgers University in 1989.

Her major research interests include: 1) steroid hormone modulation of gene expression in the brain; 2) mechanisms of neurotransmitter and neuropeptide action; and 3) developmental effects of steroids and neurotransmitters on the brain.

Currently, Dr. McCarthy's lab in the Department of Physiology, University of Maryland School of Medicine, is interested in understanding these hormonal differences and how they may contribute to neurological disorders. The major hypothesis they are testing is whether the cellular mechanism influenced by steroid hormones that results in cellular excitation is more acute and active in the male brain. If so, there might be more chances for things to go awry.

A second research project that Dr. McCarthy is

heading is looking at the effects of steroids on the incidence and outcome of cerebral vascular accidents (strokes) that occur in about 20% of premature live newborns. If such a link is established, it might then be beneficial to antagonize or block estradiol during the perinatal period.

Cheryl A. G. Scacheri, M.S.

Ms. Scacheri earned a master's degree in genetic counseling from the University of Pittsburgh and has been a diplomat of the American Board of Genetic Counseling since 1996. She serves as chair of the legislative subcommittee for the National Society of Genetic Counselors, and has a particular interest in genetic nondiscrimination. She is a faculty member at the George Washington University School of Medicine.

In her position at Children's National Medical Center, she is the coordinator of the X-chromosome inactivation study, which seeks to identify genetic causes of recurrent pregnancy loss. Ms. Scacheri is chief genetic counselor for the Muscular Dystrophy Association (MDA) Clinic at DC Children's, and her interest in neuromuscular disorders has led her to serve on the local MDA Executive Board and become a volunteer MDA camp counselor each summer.

Richard B. Simerly, Ph.D.

Dr. Simerly received an A.B. degree in zoology from the University of California at Berkeley (1976) and his Ph.D. in anatomy/neurobiology from UCLA (1984). He completed his post-doctoral training at the Salk Institute in San Diego, and was a senior research associate there before joining the faculty of the Oregon Regional Primate Research Center and Oregon Health Sciences University in 1990.

His research efforts are directed toward studying the organization and development of hormone sensitive neural pathways in the mammalian forebrain. The central goal of the laboratory is to identify signaling mechanisms that control the development and differentiation of forebrain pathways mediating neuroendocrine function, which may provide insight into the causes of hormone sensitive developmental abnormalities and neurological disorders.

John G. Vandenberg, Ph.D.

Dr. Vandenberg is a Professor of Zoology at North Carolina State. After completing his Ph.D. at Pennsylvania State University in 1962, he spent 15 years in full-time research first with the NIH in Puerto Rico and then at Dorothea Dix Hospital in Raleigh, NC. He moved to North Carolina State University in 1977 to lead the Department of Zoology through a major growth period. In 1990, he left administration to focus on teaching and managing a research program in behavioral endocrinology. His research resulted in the discovery that male mice can accelerate the onset of puberty in females through a pheromone excreted in their urine. This has become known as the "Vandenberg Effect" joining other effects on mouse reproduction such as the Bruce and Whitten effects. In recent years his research has explored whether prenatal exposure of female mice to natural hormones from their male siblings in utero can influence later reproductive and behavioral characteristics of the offspring. This led to findings that endocrine disruptors present during gestation have similar effects on the developing fetus. His work is conducted in the W.M. Keck Center for Behavioral Biology at N.C. State University.

Frederick S. vom Saal, Ph.D.

Dr. vom Saal is a professor in the division of biological sciences at the University of Missouri-Columbia. He taught biology in the Peace Corps in Somalia and Kenya after graduating from New York University. He subsequently received a Ph.D. in neuroscience at Rutgers University and postdoctoral training in reproductive physiology at the University of Texas at Austin. Dr. vom Saal is a fellow of the American Association for the Advancement of Science. His research concerns the long-term consequences of exposure during fetal life of the brain and reproductive organs to natural hormones, and both manmade and naturally occurring endocrine disrupting chemicals.

Christine K. Wagner, Ph.D.

Dr. Wagner received a Ph.D. in neuroscience from Michigan State University in 1991 following a B.A. from SUNY Albany in psychology/biology. Dr. Wagner became a faculty member in the

Neuroscience and Behavioral Program at University of Massachusetts, Amherst in 1997 and has recently moved into a faculty position in the Department of Psychology and the Center for Neuroscience Research at the University at Albany - SUNY where she teaches behavioral neuroscience and the neurobiology of mental illness.

Dr. Wagner's research program examines the role of steroid hormones in neural development, particularly the mechanisms by which steroids act to produce sex differences in the brain. Her work focusing on the role of maternal progesterone in the sexual differentiation of the brain has been funded by grants from NIMH and is currently funded by an RO1 from NICHD.

Huntington F. Willard, Ph.D.

Dr. Willard trained in genetics at Harvard, Yale, and Johns Hopkins Universities. Dr. Willard is a respected leader nationally and internationally in the field of human genetics. Prior to coming to University Hospitals of Cleveland and Case Western Reserve University in 1992, he held faculty positions at the University of Toronto and at Stanford University. Currently President of the American Society of Human Genetics, he has also served in elected leadership positions in the Association of Professors of Human/Medical Genetics, and the Human Genome Organization.

His research interests include human chromosome structure and function, X chromosome inactivation and epigenetic mechanisms of gene silencing, and development of human artificial chromosomes for studies of gene transfer and functional genomics.

In 1999, Dr. Willard was appointed Director and President of The Research Institute of University Hospitals of Cleveland. The Research Institute is among the nation's largest, with over \$75 million in funded research activities across the full spectrum of biomedical research, from fundamental investigation of biological processes to the development of new approaches to therapeutics and health care in the coming era of Genetic and Genomic Medicine.

SEX BEGINS IN THE WOMB PLANNING COMMITTEE

CO-CHAIR:

Mary Lake Polan, M.D., Ph.D., M.P.H.
Stanford University School of Medicine

CO-CHAIR:

Linda Giudice, Ph.D., M.D.
Stanford University School of Medicine

Regina Casper, M.D.
Stanford University School of Medicine

Melvin Grumbach, M.D.
University of California, San Francisco

Florence Haseltine, Ph.D., M.D.
Director, Center for Population Research
National Institute of Child Health & Human
Development
National Institutes of Health

Ellen Lovelace, M.P.H., M.A.
Stanford University School of Medicine

ABOUT THE SOCIETY FOR WOMEN'S HEALTH RESEARCH

The Society for Women's Health Research is the nation's only non-profit advocacy group whose sole mission is to improve the health of women through research. The Society was founded in 1990 when it brought to national attention the need for the appropriate inclusion of women in major medical research studies and the resulting need for greater funding for research on conditions experienced by women.

The Society initiated the groundbreaking Institute of Medicine report, *Exploring the Biological Contribution to Human Health: Does Sex Matter?* that underscored the need to better understand the importance of sex differences and how to translate that knowledge into improved medical practice and therapies.

The Society works to increase public and private funding for research on women's health, promote the inclusion of women in medical research studies, and encourage the scientific examination of the basic biological and physiological differences between men and women. The emerging field of sex-based biology explores these differences and their effect on both health and the diagnosis and treatment of disease.

HISTORY

The Society was the force behind many major advances in women's health including increased federal funding for women's health research, passage of the federal law requiring women and minorities to be included in federally funded medical research and establishment of the Office of Research on Women's Health at the National Institutes of Health. It was also responsible for the strengthened guidelines from the U.S. Food and Drug Administration to include women in all phases of drug testing. The current public awareness of gaps in women's health research is due in

large measure to the ongoing efforts of the Society.

OUTREACH

One of the Society's priorities is to promote and support the efforts of basic and clinical researchers in the emerging field of sex-based biology. The Scientific Advisory Meetings (SAMs) bring together representatives of scientific, medical, and health specialty organizations for updates on research in sex-based biology. Basic research into the molecular and cellular biology of sex differences is the focus of the Society's Annual Conferences on Sex and Gene Expression (SAGE). The Society co-sponsors a Scholars Grant Program to support the scientific and academic advancement of young physician researchers. In addition, the Society's Isis Fund for Women's Health Research sponsors collaborative networks to foster interdisciplinary basic and clinical research in areas related to improving women's health, particularly sex-based biological differences and their impact on health and disease.

The Society works with policy makers, researchers, and the public to increase public dialogue and change public policies on women's health research issues. The Society's Women's Health Research Coalition of leaders from health, medical, and scientific organizations supports increased research funding for, and expansion of, sex-based research at academic research institutions.

SOCIETY FOR WOMEN'S HEALTH RESEARCH STAFF

Phyllis Greenberger, M.S.W.

President & CEO

Anita Bollt, M.Ed.

Deputy Director, COO

Roberta Biegel, M.A.

Government Relations Director

Sherry Marts, Ph.D.

Scientific Director

L. Jo Parrish, M.A., M.B.A.

Communications & Development Director

Kirsten Blanton

Finance & Administration Coordinator

Jennifer Brindise

Communications Coordinator

Amanda Brondy

Development Assistant

Karyn Crichton

Executive Assistant/Meetings Coordinator

Sara Estes

Development Assistant

Stacey Fannon, M.S.

Programs Coordinator

Deeonna Farr

Receptionist

Sarah Gevers

Communications Manager

Jennifer Houtman

Senior Development Officer

Melissa Kaplan, M.A.

Government Relations Coordinator

Sarah Keitt, M.P.H.

Program Manager

Vicki Sitron

Program Assistant

Joi Vogin, M.S.

Senior Development Officer

Katie Wagner

Communications Coordinator

BOARD OF DIRECTORS

CHAIR

Denise Faustman, M.D., Ph.D.

Associate Professor of Medicine
Harvard Medical School
Director, Immunology Laboratory
Massachusetts General Hospital

IMMEDIATE PAST CHAIR

Gloria Sarto, M.D., Ph.D.

Professor, OB/GYN
Co-Director, National Center of
Excellence in Women's Health
University of Wisconsin

VICE CHAIR

Nanette Wenger, M.D.

Professor of Medicine,
Department of Cardiology
Emory University School of Medicine

SECRETARY/TREASURER

Irma Goertzen, R.N., M.A.

President and CEO
Magee-Women's Hospital & Research Institute

Janet Belle, R.N.

Basking Ridge, NJ

Mary Berg, Pharm.D.

Professor, College of Pharmacy
University of Iowa

Colleen Conway-Welch, R.N., Ph.D.

Professor and Dean
Vanderbilt University School of Nursing

Kathleen B. Drennan

Chief, Global Marketing &
Strategic Business Development
Global Clinical Trial Services,
an Omnicom Group Company

Gail Evans

Atlanta, GA

James R. Gavin III, M.D., Ph.D.

President and Professor of Medicine
Morehouse School of Medicine

Linda Giudice, M.D., Ph.D.

Director
Women's Health at Stanford

Florence Haseltine, Ph.D., M.D.

Bethesda, MD

Janet Henrich, M.D.

Associate Professor, Medicine and OB/GYN
Yale University School of Medicine

Ellen Leibenluft, M.D.

Clinical Associate Professor, Psychiatry
Georgetown University Medical Center

Celia Maxwell, M.D., F.A.C.P.

Assistant Vice President, Health Affairs
Director, Women's Health Institute
Howard University

Carmen Sapienza, Ph.D.

Professor
Temple University Medical School

CONTINUING MEDICAL EDUCATION POST-TEST

INSTRUCTIONS

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the ACCME through the joint sponsorship of Stanford University School of Medicine and the Society for Women's Health Research. Stanford University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians.

Stanford University School of Medicine designates this educational activity for a maximum of 2 hours of Category 1 credit toward the AMA Physicians' Recognition Award. Each physician should claim only those hours that he/she actually spends in the educational activity

Each participant who completes the post-test and achieves a passing grade of 70 percent or higher will receive an official notice indicating the number of CME hours earned. This notice should be safeguarded and may be used as documentation of earned CME hours. Participants receiving a failing grade will be notified and permitted to take a re-examination at no extra cost.

To receive credit, send completed answer sheet and \$25 (check or credit card number) to:

Stanford University School of Medicine
Office of Postgraduate Medical Education
Attention: Kari Costa, CME Course Coordinator
Alway Building, M-105
Stanford, CA 94305-5121
Fax: (650) 725-6106



The offer of continuing medical education credit for this lesson expires January 2005.

POST-TEST

1. X-inactivation is a female-specific biochemical mechanism in mammals that silences all genes on one or the other X chromosome in each cell.
True or False
2. Females are cellular mosaics, with some cells expressing alleles from the paternally inherited X chromosome and other cells expressing alleles from the maternally inherited X chromosome.
True or False
3. Genomic imprinting occurs in:
 - a. Males only
 - b. Females only
 - c. Both males and females
 - d. Rodents only
4. DNA methylation can lead to:
 - a. Gene duplication
 - b. Gene silencing
 - c. Histone acetylation
 - d. Apoptosis

5. Partial or complete loss of imprinting of *IGF2* can result in biallelic expression of IGF2, which may predispose adults and children to numerous malignancies.
True or False
6. Exposure to testosterone during fetal development of rodents results in:
 - a. Masculinization
 - b. Feminization
 - c. a and b
 - d. No change
7. Female mouse fetuses located between two males in utero grow up to be less aggressive as adults than females located between two females.
True or False
8. Male mouse fetuses located between two females in utero experience higher biologically active estradiol levels than males located between two males.
True or False
9. In rodent models prenatal stress affects fetal development in a way that:
 - a. Reduces later maternal behavior in females
 - b. Feminizes masculine behavior in males
 - c. a and b
 - d. None of the above
10. Which of the following adult onset diseases are associated with low birth weight?
 - a. Stroke
 - b. End-stage renal disease
 - c. Hypertension
 - d. All of the above
11. An increase in the number of triplet repeats in the causative gene is found in:
 - a. Rett syndrome
 - b. Fragile X syndrome
 - c. Prader-Willi syndrome
 - d. Non-syndromic X-linked mental retardation
12. Males who carry balanced translocations that can cause Down syndrome are more likely to have affected children than are females who carry the same translocations.
True or False
13. In order for manifesting carriers of Duchenne muscular dystrophy to exhibit the disease, skewed X-inactivation must occur.
True or False
14. Congenital adrenal hyperplasia exposes the fetus to lower levels of adrenal androgens throughout gestation.
True or False
15. Compared with their sisters, girls with congenital adrenal hyperplasia:
 - a. Play more with boy's toys
 - b. Play more with girl's toys
 - c. Have higher spatial ability
 - d. a and c
16. All sexually dimorphic forebrain regions contain more neurons in males than females.
True or False
17. Estrogen receptor alpha regulates the number of cells that mature in the AVPV of the hypothalamus and mediates the formation of neural pathways between these cell groups.
True or False

18. Sex differences in the brain are largely determined by:
- Presence of a Y chromosome
 - Rearing environment
 - Gonadal steroids acting on the brain during development
 - None of the above
19. What steroid hormone in addition to testosterone and its metabolites may play an important role in the sexual differentiation of the brain and behavior?
- Cortisol
 - Progesterone
 - Estradiol
 - Dihydrotestosterone
20. In rats, circulating levels of maternal progesterone do not differ between fetal males and females, rather it is the sensitivity of the developing brain to this steroid hormone that differs between the sexes.
True or False

To receive credit, send completed answer sheet and \$25 (check or credit card number) to:

Stanford University School of Medicine
Office of Postgraduate Medical Education
Attention: Kari Costa, CME Course Coordinator
Alway Building, M-105
Stanford, CA 94305-5121
Fax: (650) 725-6106

ANSWER SHEET

SEX BEGINS IN THE WOMB

Please photocopy this form for additional CME registrants. Please Print legibly.

Name _____

Affiliation _____

Address _____

City _____ State _____ Zip _____

Social Security # _____

Telephone # _____ Fax # _____

E-Mail _____

Signature _____ Date _____

PAYMENT OPTIONS:

- \$25 check enclosed (payable to Stanford University School of Medicine)
- Please charge \$25 to my credit card (Visa or MasterCard - please circle one)

Number _____ Expiration Date _____

Signature (required) _____



PLEASE INDICATE YOUR EXAM RESPONSE BY CIRCLING THE ANSWER FOR EACH.

1. True False
2. True False
3. a b c d
4. a b c d
5. True False
6. a b c d
7. True False
8. True False
9. a b c d
10. a b c d
11. a b c d
12. True False
13. True False
14. True False
15. a b c d
16. True False
17. True False
18. a b c d
19. a b c d
20. True False

LESSON EVALUATION

1. How long did it take you to complete this material? _____Minutes
2. How well did this lesson achieve its educational objectives?
 Very well
 Well
 Somewhat
 Not at all
3. How useful will the information in this lesson be to your care of patients?
 Very useful
 Useful
 Somewhat useful
 Of little use
4. What overall grade would you assign to this lesson? (Circle one.)
A B C D F
5. Did you perceive any commercial bias in this activity? Yes No



SOCIETY FOR
WOMEN'S HEALTH RESEARCH

1828 L Street, NW
Suite 625
Washington, DC 20036
202-223-8224
www.womens-health.org