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Review

Sex Differences in Cerebral Ischemia: Possible Molecular Mechanisms

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Sex is emerging as an important factor in the etiology and expression of many different pathological conditions, including stroke. Initially, the levels of sex hormones were thought to be the major contributor to these sex differences, especially after puberty, when gonadal steroid levels sharply diverge between the sexes. More recently, it is recognized that sex differences also result from the *organizational* effects of sex hormone exposure early in development, even in the absence of hormone exposure later in life, as well as effects mediated by the sex chromosomes themselves. Epigenetic modifications of developmental genes important in sexual differentiation and the response to sex steroid hormones are also emerging as another important contributor to sex differences in disease expression. This review describes recent research on the relationship between hormones, organizational-activational effects of gonadal steroids, and epigenetic modifications in brain pathology, focusing specifically on cerebral ischemia.

Key words: hormones; steroids; organizational-activational effects; epigenetics; stroke; cell death

An individual's sex can influence not only reproductive characteristics but also the function and response of nongonadal cells and tissues under both normal and stressed conditions. In recent years, interest in the influence of sex on molecular responses has increased, leading to the study of sex differences in a variety of organ systems, including the brain. In the initial part of this Mini-Review, the early studies of sex differences, which focused primarily on the role played by sex steroid hormones, will be discussed. Subsequent studies have demonstrated important contributions of the sex chromosome complement (XX vs. XY) that are independent of hormone actions that modify the response to a number of physiological and pathological stimuli. Most recently, there is growing appreciation for the influence of epigenetics and imprinting on sex differences (McCarthy et al., 2009). This Review presents evidence from several organ systems documenting the existence of sex differences, presents overviews of the emerging hypotheses on the etiology of these sex differences, then focuses on the mechanisms of sex differences in the response to cerebral ischemia.

ORGANIZATIONAL-ACTIVATIONAL HYPOTHESIS OF SEX DIFFERENCES

The study of sex differences has evolved with a central theme revolving around the organizational-activational hypothesis. The *organizational* effects of gonadal hormones are those that occur at a particular time, such as during development, and that persist even after the hormonal exposure is removed (Fig. 1). An example of this would be prenatal exposure to androgens during sex differentiation, which irreversibly commits the previously undifferentiated gonad to more masculinized genitalia. Treatment with the antiandrogen flutamide during this critical period of development alters sex organ differentiation (Herman et al., 2000). The *activational* effects are those that are reversible and that persist only while the sex steroid hormone is present (Fig. 1). For example, the secretion of acetylcholine in the dorsal hippocampus is higher in male rats compared with females. When the gonads of both sexes were surgically removed, the sex difference was ablated but could be subsequently restored by hormonal replacement (Mitsushima et al., 2009), suggesting that these differences resulted from the activational effects of hormones.

Sex differences attributed to activational and organizational effects of hormones are seen in multiple organ systems (Table I), ranging from kidney and heart (Mabley et al., 2005; Hutchens et al., 2008) to bone and brain (Hogervorst et al., 2003; Rapp et al., 2003; Karasik and

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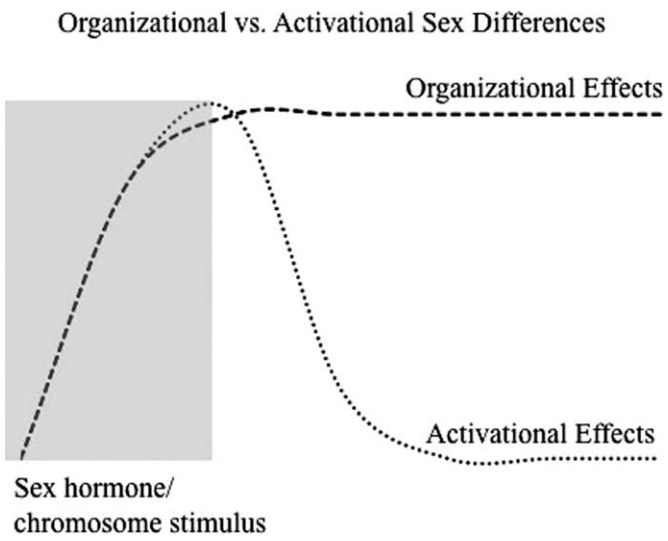

Fig. 1. Organizational vs. activational hypothesis. Organizational effects are defined by prenatal exposure to sex hormones that cause changes or activate behaviors that persist into adulthood. Activational effects are defined by changes or behaviors that persist only in the presence of the sex hormone.

Ferrari, 2008; Pietschmann et al., 2009). Multiple neurological disorders, including Alzheimer's disease (AD), multiple sclerosis (MS), and stroke, also display differences between the sexes (Table IIA,B). For instance, MS incidence is fourfold higher in women (Nicot, 2009). Women are much more likely to have the relapsing-remitting form of the disease, whereas men are more likely to be diagnosed with progressive multiple sclerosis (Whitacre et al., 1999; Pozzilli et al., 2003). This suggests that both disease incidence and virulence can be influenced by sex. Although the organizational and ativational effects of hormones explain some observed sex differences, an organism's genetic sex may also contribute.

Because distinguishing between hormonal and genetic sex effects can be difficult, the four core genotypes (FCG) mouse model was developed (Fig. 2). In this model, gonadal sex and sex chromosome complement are uncoupled. In the FCG model, Sry (testis-determining factor) has been deleted from the Y chromosome and inserted as a transgene on an autosome instead. Therefore, only the autosome determines sex, and the sex chromosomes are irrelevant to gonadal sex (De Vries et al., 2002). The FCG model results in the following four groups of mice: XX gonadal females, XY gonadal females (since there is no Sry on the Y chromosome), XX gonadal males (which have inherited Sry on an autosome), and XY gonadal males (which lack Sry on the Y chromosome but have Sry on an autosome). These four groups of mice can then be compared to examine the influence of sex chromosomes (to compare XY males vs. XX males or XX females vs. XY females) vs. gonadal hormones (to compare XY males vs. XY females or XX males vs. XX females) (Jazin and Cahill, 2010).

Several studies utilizing the FCG mice have demonstrated the importance of sex chromosome complement t. With the FCG model, it has been shown that dopaminergic neuron differentiation is greater in XY compared with XX cultures from E14.5 mice, a time at which sex hormone levels are similar in both sexes (Carruth et al., 2002). Gonadectomized XX mice have a greater responsiveness to thermal and chemical stimuli than XY mice regardless of gonadal sex (Gioiosa et al., 2008a,b). In an experimental model of MS, XX mice have a more severe and progressive disease than XY mice (Smith-Bouvier et al., 2008), suggesting that some of the epidemiological differences seen in MS patients are independent of hormones. In studies of addiction, XX mice of either gonadal sex developed habitual behaviors at a faster rate than XY mice of either gonadal sex (Quinn et al., 2007). These studies provide clear evidence that the sex chromosomes play an important role in defining phenotypic sex differences (Arnold, 2009).

The contribution of sex chromosomes has been integrated into the organizational-activational hypothesis, forming a new model for the study of sex differences. This expanded hypothesis stresses the importance of hormonal effects, both organizational (permanent) and activational (reversible), while stating that XX and XY cells differ prior to encountering gonadal hormones (Arnold, 2009). Although this theory seems to cover many bases, is it a complete theory to define sex differences? Are there any factors modulating gene expression that may contribute to different phenotypes between the sexes?

EPIGENETICS OF SEX DIFFERENCES

Phenotypic differences can also result from epigenetic changes, which can occur by methylation of DNA or alterations to histone proteins, which package DNA into chromatin. DNA methylation and histone deacetylation generally inhibit gene expression, whereas histone acetylation leads to increased gene expression (McCarthy et al., 2009). A classic example of epigenetic changes relevant to sex differences is the random inactivation of one of the two X chromosomes in females, which occurs early in development. However, in humans, as many as 15% of genes located on the second X chromosome always escape inactivation, and an additional 10% sometimes escape inactivation (Carrel and Willard, 2005). This suggests a remarkable and previously unsuspected degree of expression heterogeneity among females. Therefore, it is possible that sex-specific traits may be mediated by gene dosage. Adding another level of complexity is that female individuals are mosaic, in that, during female development, cell lineages randomly inactivate their paternal X or their maternal X, leading to possible genetic imprinting effects (Migeon, 2007). This epigenetic complexity can have consequences in disease expression, as demonstrated in the OXVASC study, which observed a higher risk of stroke in women with a maternal history of stroke (Touze, 2007).

TABLE I. Sex Differences in Different Organ Systems*

Author	Model/organ system	Type of sex difference	Effect
Chen et al., 2003	Cardiac ischemia	Mixed	Mechanism of female protection due to less calcium overload in females
Corsi et al., 2007	Osteoporosis	Unknown	Male skeletal muscle derived stem cells had higher osteogenic gene expression and alkaline phosphatase activity than female cells when treated with BMP4
Cross et al., 2003	Cardiac ischemia	Mixed	PLB KO increased ischemic injury in both sexes, but female hearts were less susceptible
Gabel et al., 2005	Cardiac ischemia	Mixed	Estrogen protection occurs through ERβ and females had an increased ratio of carbohydrate to fatty acid metabolism than males
Garnero et al., 2000	Osteoporosis	Unknown	Osteoporosis and fracture were more common in women and were related to ovarian hormone levels
Gilliver et al., 2008	Wound healing	Mixed	Wounds were larger in ovariectomized females compared to castrated males
Hanada et al., 2009	Conditional RANK KO	Unknown	RANK controlled thermoregulation in females
Kim et al., 2009	Silver nanoparticle delivery	Unknown	Ag nanoparticles accumulated at a twofold higher concentration in female kidneys
Krishnan et al., 2009	Sex differences in development	Organizational	The anteroventral periventricular nucleus was smaller and had increased apoptotic proteins in males during development
Lin et al., 2009	Stress	Unknown	CREB and pCREB expression was reduced by stress in males; males recovered, whereas females did not
Mabley et al., 2005	Cardiovascular inflammation	Mixed	Endotoxin treatment increased vascular response in males compared with females, and ovariectomy partially reversed the protection
Muller et al., 2002	Renal ischemia	Activational	Females have improved survival; estrogen conferred protection, whereas ovariectomy and testosterone were ineffective
Park et al., 2004	Renal ischemia	Activational	Males had significantly greater injury, and androgen treatment increased injury
Philips et al., 2010	Stress	Unknown	Decreased anxiety in males and increased anxiety in female Myg-1 knockouts
Schwindinger et al., 2009	Diet-induced obesity	Unknown	Female mice had decreased weight gain and fat pad mass compared with males in Gng3 KO
Wang et al., 2005	Cardiac ischemia	Unknown	Female hearts were protected because of a decreased inflammatory response
Yang et al., 2005	Osteoporosis	Unknown	Vertebral strength decreased significantly earlier in females compared with males

*The types of sex differences are: Organizational (due to the effects of prenatal hormone exposure or sex chromosomes), Activational (due to the presence of a sex hormone after puberty and effect disappears if hormone is removed), Mixed (effect may be modulated by sex hormones), and Unknown (unable to discern direct mechanism of sex differences). *Abbreviations*: BMP4, Bone Morphogenetic Protein 4; PLB, Phospholamban; ER-beta, Estrogen Receptor-beta; RANK, Receptor Activator of Nuclear Factor κB; CREB, Cyclin Adenosine Monophosphate Response Element Binding Protein; Myg-1, Melanocyte Proliferating Gene 1; Gng3, G protein gamma 3 subunit.

Epigenetic modifications can also directly influence the response to sex steroid hormones, insofar as estrogen receptor α (ERα), but not estrogen receptor β, undergoes epigenetic changes. The ERα promoter is methylated in the aging colon and in some cancers (Issa et al., 1994; Ottaviano et al., 1994; Sasaki et al., 2002). During development, estrogen acts via ERα, altering gene expression. In the rodent cortex, ERα is highly expressed early in postnatal development, sharply declines by PD10, and remains relatively low through adulthood (Westberry et al., 2010). Sex differences in ERα methylation occur in the preoptic area and hypothalamus, two sexually dimorphic brain regions involved in the development of adult sexual behavior. Early in development, mothers groom male pups in the anogenital region more than female pups. During this critical period, males have more *overall* ERα methylation than females (Auger and Olesen, 2009), potentially reducing ER signaling. However, methylation status may vary depending on the brain region examined, insofar as females have more ERα and progesterone receptor methylation than males in the developing hypothalamus (McCarthy et al., 2009). Differences in epigenetic modifications also appear to play a role in the response to cerebral ischemia. ERα mRNA levels are significantly higher in females compared with males after stroke, and, interestingly, there is *decreased* methylation of the ERα promoter in females but not in males after an ischemic challenge (Westberry et al., 2008; Wilson et al., 2008). This suggests that stroke-induced demethylation of the ERα promoter occurs preferentially in female brain, leading to an increase in ERα expression and estrogen signaling. The neuroprotective effect of estrogen is mediated by ERα. Both male and female neuronal ERα receptor knockout mice have larger strokes compared with wild-type mice and lose the neuroprotective effects of exogenous estrogen (Dubal et al., 2006; Elzer et al., 2009). It is possible that acute demethylation of the ERα promoter contributes to the neuroprotective effects of estrogen, especially in the female brain.

Methylation also occurs in the promoter of the androgen receptor (AR), as seen in 20–30% of prostate tumors (Kinoshita et al., 2000) and in patients with premature puberty (Leader et al., 2006). This suggests that AR methylation may also play an important role in the pathology of different diseases, but this has not been investigated in ischemic brain.

Changes in histone acetylation also contribute to sex differences in the brain. Under normal conditions, females

TABLE II. Sex Differences in Neurological Disorders and Cerebral Ischemia*

Author	Neurological disorder or ischemic model	Type of sex difference	Effect
A.			
Baldereschi et al., 2000	Parkinson's disease	Unknown	Males had a significantly higher incidence of Parkinson's disease than females
Bode et al., 2008	Huntington's disease	Unknown	tgHD male rats displayed impairment of motor function associated with decreased striatal D1 receptor density and decreased estradiol levels compared with tgHD female rats
Hauben et al., 2002	Spinal cord injury	Unknown	Females recovered better than males secondary to a beneficial immune response in females, which may be blunted in males by testoserone
Hogervorst et al., 2004	Alzheimer's disease	Unknown	Low free testosterone was a risk factor for Alzheimer's disease
Morissette et al., 2008	Parkinson's disease	Unknown	Estrogen was neuroprotective by interactions with PI3K, Akt, GSK3β, Bcl-2, and BAD
Pozzilli et al., 2003	Multiple sclerosis	Unknown	Men develop less inflammatory, but more destructive lesions than women
Slewa-Younan et al., 2004	Traumatic brain injury	Unknown	Severity in men was greater than that in women despite equivalent levels of injury
Spach et al., 2009	Experimental Autoimmune Encephalomyelitis	Mixed	Female disease severity remained constant, while male disease severity increased with age due in part to the Y chromosome
Tsolaki et al., 2005	Alzheimer's disease	Unknown	Females with Alzheimer's disease had significantly less estrogen than healthy females
B.			
Alkayed et al., 1998	Adult rat MCAo	Activational	Female rats had decreased stroke volumes compared with males and ovariectomized females
Hagberg et al., 2004	Neonatal HI	Organizational	PARP-1 knockout was neuroprotective in males but not females
Liu et al., 2009	Adult mouse MCAo	Activational	Caspase inhibition with Q-VD-OPH protected intact and ovariectomized females but not males
Nijboer et al., 2007	Neonatal HI	Organizational	2-Immunobiotin was protective in females caused by reduced HSP70 and cytochrome-c
Renolleau et al., 2007	Neonatal HI	Organizational	Caspase inhibition by Q-VD-OPH protected females but not males
Yuan et al., 2009	Adult mouse MCAo	Unknown	PARP-1 deletion, decreased PAR formation, and AIF down-regulation preferentially protected males compared to females
Zhu et al., 2006	Neonatal HI	Organizational	Females experienced increased caspase activation, while males experienced increased AIF translocation

*The types of sex differences are organizational (resulting from the effects of prenatal hormone exposure or sex chromosomes), activational (resulting from the presence of a sex hormone after puberty and effect disappears if hormone is removed), mixed (effect may be modulated by sex hormones), and unknown (unable to discern direct mechanism of sex differences). *Abbreviations*: MCAo, Middle Cerebral Artery Occulstion; HI, Hypoxia Ischemia; tgHD, Transgenic Huntington Disease; PI3K, Phosphoinositide 3 Kinase; Akt, Protein Kinase B; GSK3-beta, Glycogen Synthase Kinase 3-beta, Bcl-2, B Cell Lymphoma 2; BAD, Bcl-2 Associated Death Promoter; PARP-1, Poly ADP-Ribose Polymerase 1; HSP70, Heat Shock Protein 70; PAR, Poly ADP Ribose Polymer; AIF, Apoptosis Inducing Factor.

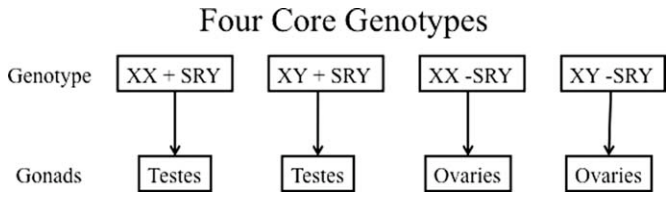

Fig. 2. Four core genotypes (FCG) animal model. This animal model was developed in order to separate sex hormone and sex chromosome effects. The SRY gene is removed from the Y chromosome and inserted on an autosome, making four different genotypes possible. The four core genotypes are an XX female, XY (no autosomal SRY) female, XX (+ autosomal SRY) male, and XY (+ autosomal SRY) male. Modified from Jazin and Cahill (2010).

have less histone H3 acetylation, leading to decreased transcription compared with males in the cortex and hippocampus. This is ameliorated by treatment with testosterone, which increases acetylation and transcription in females. This demonstrates that hormones can act in concert with epigenetic modifications to shape neural development (Tsai et al., 2009). Interestingly, steroid hormone receptor coactivators have histone acetylation activity or recruit histone acetylases. Inhibition of histone deacetylation leads to a repression of genes important in masculinization of the bed nucleus of the stria terminalis, a sexually dimorphic brain region (McCarthy et al., 2009). Similarly, both ERα and AR are acetylated by the histone acetyltransferase p300, and ERα is deacetylated by histone deacetylase-1 (HDAC-1; Leader et al., 2006). Therefore, epigenetic modifications play an important role in the expression of steroid receptors and other gene targets that are involved in the development of "sex differences." This Review will focus now on cerebral ischemia, in which sex differences exist in both preclinical and clinical populations (Lang and McCullough, 2008; Turtzo and McCullough, 2008), and explores the possible role of epigenetics, hormones, and sex chromosomes in the sexual dimorphism seen in stroke.

SEX DIFFERENCES IN STROKE

Stroke is the third leading cause of death in the United States and the leading cause of long-term disability. The incidence of stroke in women is lower than in men until decades past menopause, suggesting that factors beyond ovarian hormone exposure contribute to these epidemiological sex differences (Rosamond et al., 2008; Turtzo and McCullough, 2008). Hormone replacement therapy has been ineffective for both primary and secondary stroke prevention, and chronic estrogen use is associated with higher rates of stroke in treated women (Wassertheil-Smoller et al., 2003; Anderson et al., 2004). This suggests that the replacement of ovarian hormones does not replicate the intrinsic female protection seen throughout much of a woman's life span. Even in neonatal populations, when there are minimal differences in hormone levels between the sexes, males appear to have an ''ischemia-sensitive'' phenotype (Zhu et al., 2006; Johnston and Hagberg, 2007). Not until women are over age 80 years does their incidence of stroke surpass that of men, suggesting that nonhormonal factors play a role in ischemic sensitivity. These clinical results suggest that not all of the protection afforded to females following ischemic insult is hormonally mediated, a hypothesis that is further supported by recent laboratory stroke studies. Investigation into the mechanisms of cell death has led researchers to believe that the process of cell death through apoptosis is activated differentially in male and female cells (Table IIB).

Work in our laboratory and by others demonstrates striking sex differences in the cell death pathways triggered by an ischemic insult (Du et al., 2004; Hagberg et al., 2004; McCullough et al., 2005; Renolleau et al., 2007; Li and McCullough, 2009; Liu et al., 2009; Yuan et al., 2009). Although cell death occurs after experimental stroke in both sexes, the response to the injury is different, and these patterns are set in early development (Rosen et al., 1999; Hagberg et al., 2004; Zhu et al., 2006; Renolleau et al., 2007). Why could this have therapeutic relevance? To date, despite a number of promising preclinical strategies, all clinical trials of putative neuroprotective agents have failed. This suggests that our understanding of cell death in the ischemic brain is far from complete. Sex differences must be mechanistically defined in order to understand better where, and in whom, neuroprotective therapies will be beneficial. The development of sex-specific therapies may improve our ability to treat stroke patients.

STROKE INDUCES CELL DEATH

Ischemic cell death is triggered by an influx of calcium, with subsequent oxidative damage and mitochondrial dysfunction, activating several distinct cell death pathways. The immediate energy failure seen in the ischemic core leads to swelling of the mitochondria and loss of membrane integrity, typical of necrotic cell death (Yuan et al., 2009). Reducing necrotic cell death is extremely difficult and likely requires reperfusion early during the ischemic insult. However, a more delayed and controlled apoptotic cell death also occurs in the ischemic brain. The cell death pathways triggering apoptosis have been well studied, because these represent an attractive target for therapeutic intervention. Over the past decade, both caspase-dependent and caspase-independent cell death pathways have been recognized, adding considerable complexity to studies of ischemic cell death (Yu et al., 2002). Interestingly, organizational-activational effects and epigenetic regulation have not been a focus of stroke research to date. This Review links these broad hypotheses to the current literature in an attempt to integrate possible mechanisms by which sex differences in cerebral ischemia occur.

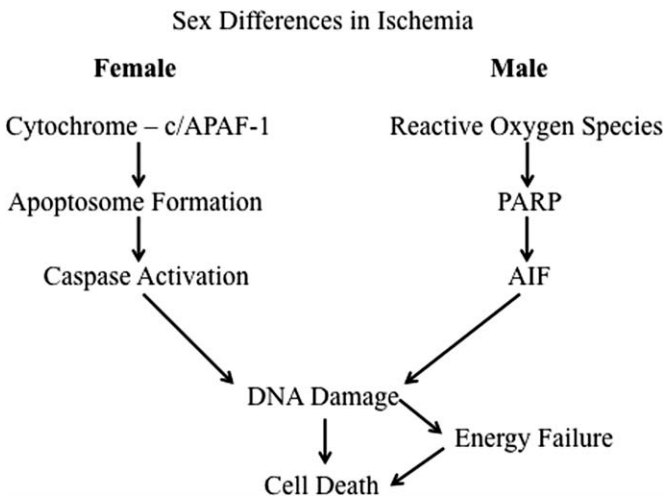

Fig. 3. Model of sex differences in ischemic cell death pathways. Ischemic cell death is thought to occur predominantly by caspase-mediated mechanisms in females and caspase-independent mechanisms in males.

POLY-ADP RIBOSE-INDUCED CELL DEATH

The DNA repair enzyme poly-ADP-ribose polymerase (PARP-1) induces one well-recognized mechanism of caspase-independent apoptotic death via neuronal nitric oxide synthase (nNOS), PARP-1, and apoptosis-inducing factor (AIF), which plays a key role in regulating cell death in the male brain (for review see Lang and McCullough, 2008; Fig. 3). Cell death induced by activation of nNOS and PARP is clearly sexually dimorphic.

Although nNOS deletion and inhibition were neuroprotective in adult male mice, these manipulations exacerbated stroke damage in adult female mice (McCullough et al., 2005). This sexually dimorphic response was unaffected by hormone exposure/replacement, suggesting that it was either hormone-independent (a sex-specific effect) or secondary to organizational or epigenetic effects induced by early gonadal steroid exposure Several studies provide evidence that this sex-specific ischemic sensitivity occurs early in development, prior to the activational effects of steroids at puberty. PARP-1 gene deletion protected male pups in a neonatal hypoxic-ischemic injury (HI) model but had no effects on female pups, (Hagberg et al., 2004), and

HI preferentially activated PARP-1 signaling in P9 male mice as seen by enhanced PARP-1-activated translocation of AIF and subsequent caspase-independent cell death (Zhu et al., 2006) compared with females. In contrast, female P9 mice demonstrated significantly more caspase-3 activity after HI than males, implying activation of caspase-induced cell death pathways. Interestingly, this is somewhat different from what occurs in adult animals. Adult females sustain *greater* damage when PARP-1 is absent (either through genetic deletion or with pharmacological inhibition), suggesting that activational effects or aging itself amplify these sex differences. This exacerbation of injury in adult females when PARP is absent appears to be independent of apparent activation of the PARP-1/AIF pathway (Yuan et al., 2009), inasmuch as manipulating the levels of PARP-1 or AIF did not correlate with infarct size in the female brain (McCullough et al., 2005).

The mechanisms by which sex differences in PARP-1 mediated-cell death occur are unknown. As seen in neonatal studies, organizational effects play a role. PARP-1 can interact with the testis-determining factor SRY (Li et al., 2006), inhibiting SRY's ability to form DNA complexes to enhance gene transcription. The mechanism by which this occurs is currently unknown, but several hypotheses exist. PARP-1 may bind to the DNA-binding domain of the SRY gene, or PARP-1 may ribosylate SRY, decreasing its ability to bind to potential target genes (Li et al., 2006). The exacerbation of ischemic injury in PARP-1 knockout female mice may be due to an organizational effect caused by loss of inhibition of the SRY gene activation, leading to a more masculinized ischemic sensitivity pattern.

The possible clinical relevance of these findings becomes clear when one considers that minocycline, a putative PARP-1 inhibitor, is currently in clinical trial for treatment of stroke (Lampl et al., 2007). If this agent were protecting via PARP-1 inhibition, we would not expect it to be efficacious in females. Review of the literature revealed that no published preclinical trials had been performed in females. Subsequent work in our laboratory demonstrated that minocycline did not protect ovariectomized female mice despite a robust reduction in infarct in males (Li and McCullough, 2009). Interestingly, PAR polymer formation was reduced equivalently in males and females given minocycline. This again suggests that the relationship between PAR and infarct is nonlinear, at least in females. The relevance of this to clinical populations, if any, is not yet known, but this should serve as a cautionary note for those investigators designing pharmaceutical trials.

ALTERNATIVE PATHS TO CELL DEATH: CASPASES

Unlike the dramatic neuroprotection seen in males with inhibition of the NO-PARP-1-AIF cell death pathway, females had an exacerbation of injury (Yuan et al., 2009). If females are insensitive to the cell death induced by PARP-1 and AIF, then what triggers apoptosis in the female brain? New evidence suggests that caspases may mediate injury in the female brain after an ischemic insult (Liu et al., 2009), although this pathway clearly can occur in both sexes (Le et al., 2002; Fig. 3). The best evidence for females' intrinsic sensitivity to caspase-mediated cell death comes from studies utilizing the selective pan-caspase inhibitor QVD-OPH, which is neuroprotective in both neonatal and adult female mice after ischemic injury but has no effect in males (Renolleau et al., 2007; Liu et al., 2009). The neuroprotective effect of QVD-OPH in females is independent of estrogen (Liu et al., 2009), suggesting either an organizational or an epigenetic difference between males and females with regard to caspase-mediated cell death.

ORGANIZATIONAL-ACTIVATIONAL, SEX CHROMOSOME, AND EPIGENETIC INFLUENCES IN CELL DEATH

The influence of organizational-activational effects of hormones and sex chromosome complement in cell death pathways is also apparent in vitro. Neuronal cultures have been obtained from prenatal animals, in which hormone levels are low but in which organizational effects of hormones have already occurred during sex determination. XX cortical neurons were more sensitive to apoptotic inducers (etoposide and staurosporine), and neurons derived from XY embryos were more sensitive to nitrosative and excitotoxic damage ($ONOO^-$ and glutamate; Du et al., 2004). Female-derived neurons also showed early mitochondrial release of cytochrome C, the hallmark of activation of the caspase cascade and oligonucleosomal DNA fragmentation patterns, all consistent with primary caspase-mediated cell death. XY-derived neurons exhibited the classic 50-kb fragmentation seen with PARP-1/AIF pathway activation and noncaspase-mediated cell death pathways. Because the cells in these studies were grown in the absence of sex steroid hormones and hormone mimics, the sex differences in preference for PARP-1/AIF vs. caspase-mediated cell death pathways are the result of organizational effects, sex chromosome complement, or a combination. Whether the differences in DNA fragmentation pattern result from epigenetic differences in DNA structure between males and females is currently unknown.

Additional evidence for the contributions of genetic sex and epigenetics in stroke comes from the clinical literature. Women with Turner's syndrome who have only one X chromosome (XO) suffer from an increased risk of ischemic heart disease, hypertension, and stroke (Gravholt, 2001). This increased stroke risk may result from the lack of a second X chromosome, but interpretation is complicated by the high rate of premature ovarian failure in XO women, suggesting a role for estrogen as well as well as the high degree of mosaicism seen in these women, with some having a mixed XX/XO cell karyotype.

NOVEL CELL DEATH PATHWAYS ALSO SHOW SEXUAL DIMORPHISM

A novel form of cell death that has recently come under study by stroke researchers, autophagy, also

appears to exhibit sex differences. Autophagy involves sequestering pieces of the cell's cytoplasm into an autophagosome destined for degradation by the lysosome (Shintani and Klionsky, 2004), allowing for recycling of cellular contents. Autophagic cell death occurs after neonatal HI in males, as shown by increases in autophagosome formation and lysosome activity, mainly in the penumbra. Autophagic cell death inhibitors are neuroprotective in this model, decreasing ischemic damage (Puyal et al., 2009). Intriguingly, in sex-specific neuronal cultures challenged with nutrient deprivation, male-derived neurons readily undergo autophagy and die, whereas female-derived neurons mobilized fatty acids, accumulated triglycerides, formed lipid droplets, and survived longer. Importantly, autophagic inhibitors preferentially protected male-derived neurons, although it is not yet known whether these sex differences are also seen in vivo. Therefore, sex differences are present not just in PARP-1 or caspase pathways but also in other, diverse cell death mechanisms. Similarly, not all sex differences are related to ischemia or stress, nor are they seen exclusively in the brain (Du et al., 2009).

WHAT ABOUT HORMONES?

The activational effects of sex steroid hormones on stroke are best illustrated by animal studies of estrogen. Ovariectomized female mice have increased stroke volume compared with females with intact ovarian function (Alkayed et al., 1998, 2000; Hurn and Macrae, 2000; Sawada et al., 2000). Treatment with estrogen is neuroprotective in most animal models, and estrogen reduces infarct size when acutely administered to males, ovariectomized females, and reproductively senescent females (McCullough and Hurn, 2003). Although estrogen confers protection in experimental stroke, clinical translation of estrogen's protective effect has been unsuccessful to date (Turtzo and McCullough, 2008).

Recently, the role of testosterone in ischemic sensitivity has been investigated. Clinically, elevated endogenous testosterone correlates with increased stroke risk in young boys, but not in young girls (Normann et al., 2009; Vannucci and Hurn, 2009). In animals, low doses of testosterone administered to castrates decreased infarction volume, whereas higher doses exacerbated infarction volume. The androgen receptor antagonist flutamide blocked these effects, suggesting a key role of the AR in mediating the ischemic response to testosterone (Uchida et al., 2009). P0 female rats masculinized with dihydrotestosterone (DHT), an androgen receptor agonist, exhibited increased damage in the hippocampus, which was associated with decreased clearance of intracellular Ca^{2+} (Nunez and McCarthy, 2008). These results suggest that testosterone is a contributor to the ''ischemia-sensitive'' phenotype seen in males.

Extragonadal (i.e., brain) hormone production can also influence stroke sensitivity. In females, absence of aromatase, the enzyme that converts androgens to estrogens, increased infarction volumes. Interestingly, ovariectomized females (with loss of gonadal estrogen) had smaller strokes than aromatase knockout animals, suggesting that extragonadal hormone production also can protect against ischemic cell death (McCullough et al., 2003). In vitro, female astrocytes are protected from oxygen glucose deprivation compared with male-derived astrocytes and have higher levels of aromatase activity. Pharmacological inhibition of aromatase eliminates this intrinsic female neuroprotection (Liu et al., 2007), suggesting that the differential production of estradiol by aromatization between the sexes plays an important role in the sex differences in ischemic cell death (Roselli et al., 2009).

WHERE DO WE STAND?

The recent literature suggests that sex differences exist in ischemic cell death pathways. Hormonal contributions to any sexually dimorphic phenotype must be considered and may result from brief periods of exposure during critical times in development. If hormones play a role in the response to ischemic injury, these effects must occur early in development, because sex differences in sensitivity to NO-mediated cell death (Nijboer et al., 2007) are seen as early as postnatal day 3. Ongoing studies are examining the effect of prenatal and neonatal hormone exposure (i.e., testosterone to females or androgen antagonists to males) to determine whether this early ischemic sensitivity can be reversed. Androgen exposure will likely enhance a female's sensitivity to ischemia, but will treatment also reverse the predominant cell death pathway from caspases to NO/PARP-1? One way to evaluate this is to masculinize female PARP-1 knockouts, which have larger infarcts than WT females at baseline. If cell death patterns can be ''rewired,'' then one would expect to see a reduction in infarction volume in these masculinized PARP-1 knockout females similar to that in male PARP-1 knockout mice. In addition, phenotypic changes that are seen with acute or developmental hormone exposure are influenced by the genotypic environment or biologic sex (XX or XY). The FCG model (Fig. 2) would be an excellent way to approach the organizational vs. activational effects of hormones in cerebral ischemia. This would help stroke researchers to determine the mechanism by which sex differences occur.

What are the origins of nonhormonally mediated sex differences in cell death mechanisms? As described above, strong cases exist for the involvement of the organizational-activational hypothesis, imprinting/epigenetics, and sex hormones in sex differences in cell death. It is important to remember that these are not mutually exclusive ideas. These hypotheses encompass almost all of what is known about sex differences to date. In any disease, it is paramount to consider whether sex differences exist. At the same time, it is imperative to determine whether any or all of the three main causes of sex differences are involved (the organizational-activational hypothesis, epigenetics, and sex hormones; Fig. 4).

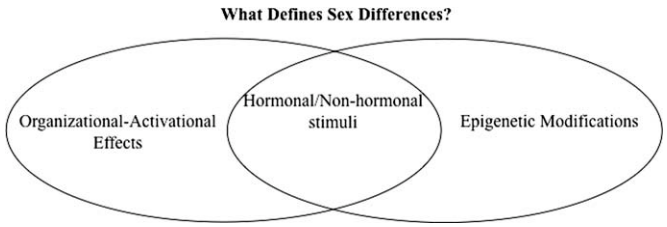

Fig. 4. Summary of the major possible contributors to sex differences. Both epigenetic modifications and the organizational-activational effects lead to sex differences. Although they are separate mechanisms, their paths cross as the mechanism by which both occur may be hormonal or nonhormonal. All must be considered when studying sex differences.

Most importantly, although these are fascinating scientific questions, one must consider the translational relevance of these sex differences, which are likely also present in other neurological diseases. Many researchers are unaware of the potential confounding effects of sex differences, and much of the preclinical work in stroke continues to focus on young male animals despite the Stroke Therapy Academy Industry Roundtable (STAIR) recommendations that neuroprotective studies be performed in both male and female rodents (Fisher et al., 2009). It is imperative that investigators become aware of the potential for erroneous conclusions when attempting to translate promising experimental findings based solely on preclinical data on one sex into a clinical population, which includes both sexes. Advancing our knowledge of the mechanisms of ischemic cell death and neuroprotective therapies is an important goal in both sexes in order to optimize treatments for stroke, and it is likely that sex-specific therapies are on the horizon.

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