Brief Communications

X Chromosome Dosage and the Response to Cerebral Ischemia

L. Christine Turtzo,[1,2,3,4] **Chad Siegel,**[1,2] **and Louise D. McCullough**[1,2]

Departments of [1]Neurology and [2]Neuroscience, University of Connecticut Health Center, Farmington, Connecticut 06030, [3]Center for Neuroscience and Regenerative Medicine, Henry M. Jackson Foundation, Rockville, Maryland 20852, and [4]Laboratory of Diagnostic Radiology Research, Clinical Center, National Institutes of Health, Bethesda, Maryland 20892

Gonadal hormones contribute to ischemic neuroprotection, but cannot fully explain the observed sexual dimorphism in stroke outcomes seen during life stages with low sex steroid hormones. Sex chromosomal complement (XX in females; XY in males) may also contribute to ischemic sexual dimorphism. A transient middle cerebral artery occlusion model was used to investigate the role of X chromosome dosage in female XX and XO littermates of two mouse strains (*Paf* and *Eda*[Ta]). Cohorts of XX and XO gonadally intact, ovariectomized, and ovariectomized females supplemented with estrogen were examined. Infarct sizes were equivalent between ovariectomized XX and XO mice, between intact XX and XO mice, and between estrogen-supplemented ovariectomized XX and XO mice. This is the first study to investigate the role of sex chromosome dosage in the response to cerebral ischemia. Neither the number of X chromosomes nor the parent of origin of the remaining X chromosome had a significant effect on the degree of cerebral infarction after experimental stroke in adult female mice. Estrogen was protective against cerebral ischemia in both XX and XO mice.

Introduction

Sex differences in both animal models of stroke and in clinical populations are increasingly recognized (for review, see Bushnell, 2008; Reeves et al., 2008; Turtzo and McCullough, 2010). The incidence of stroke in women increases at the time of menopause, but does not surpass that of men until after the age of 85 (Lloyd-Jones et al., 2009). The underlying etiology of this sexual dimorphism has been largely attributed to exposure to ovarian hormones, particularly estrogen (Herson et al., 2009). In rodent models, females have smaller infarcts than males when estrogen is present (Alkayed et al., 1998), an effect that is reversed with ovariectomy and restored with estrogen replacement (Alkayed et al., 2000). Estrogens are also neuroprotective in males (McCullough et al., 2001) and in middle-aged animals (Liu et al., 2009). However, clinical trials of postmenopausal estrogen replacement showed an increased ischemic stroke risk in treated women (Hulley et al., 1998; Viscoli et al., 2001; Wassertheil-Smoller et al., 2003). While sex steroids clearly have an effect on damage in cerebral ischemia, evidence is emerging that there are intrinsic sex differences independent of hormonal status (for review, see Turtzo and McCullough, 2010). Data from both cell culture of immature neurons (Du et al., 2004) and *in vivo* studies of neonatal animals (Renolleau et al., 2007, 2008) indicate that mecha-

nisms of cell death differ between males and females even in the absence of hormones (for review, see Lang and McCullough, 2008). Expression of both autosomal and sex chromosome-linked genes in the mouse brain displays regional sexual dimorphism, as well as sex-specific imprinting from the parent of origin (Gregg et al., 2010). In clinical studies, childhood ischemic stroke is more common in boys than in girls, both in preadolescents and adolescents, suggesting a greater influence of sex chromosomes than gonadal hormones in this population (Golomb et al., 2009), although exposure to testosterone could also be a factor.

To date, no experimental studies have investigated the contribution of sex chromosome complement to outcomes after cerebral ischemia. The hypothesis that the dosage of X chromosomes influences the response to cerebral ischemia was tested, with females predicted to have an advantage over males secondary to their additional X chromosome. To address this, ischemic sensitivity was examined in two strains of mice where a proportion of females lack the second X chromosome.

Materials and Methods

Experimental animals. The present study was conducted in accordance with the NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the University of Connecticut Health Center. *Paf/Y* males and *Paf/+* females were originally purchased from the Jackson Laboratory to establish a breeding colony, from which *Paf/+* (XX), *Paf/Paf* (XX), and *Paf/O* (XO) females and *Paf/Y* (XY) and wild-type (XY) males were generated and distinguished by coat phenotype (Lane and Davisson, 1990). The *Paf* mutation is associated with an X chromosome inversion spanning the pseudoautosomal region boundary (Burgoyne and Evans, 2000). As a result of this inversion, *Paf* males have an increased frequency of meiotic nondisjunction, resulting in high levels of sperm with abnormal numbers of sex chromosomes, and father 39 XO female progeny at a frequency as high as 20% (Raefski and O'Neill, 2005). Hemizygous females (*Paf*/O;

XO) can be identified by their distinctive patchy hair loss, while their heterozygous female (*Paf*/+; XX) littermates have striped fur patterns (Lane and Davisson, 1990). Aside from coat pattern differences, these *Paf* XO females have no described abnormalities, and are fertile.

Eda^{Ta}/+ (XX) and *Eda^{Ta}*/+O (XO) female mice were purchased from the Jackson Laboratory(Probst et al., 2008). The *Eda^{Ta}* XO mouse, which was originally described as the X-linked tabby (Ta) mutation (Falconer, 1952), results from a 2 kb deletion at the 5′ end of the ectodysplasin A (*Eda*) gene (Ferguson et al., 1997; Srivastava et al., 1997). In contrast to *Paf* XO mice, *Eda^{Ta}* XO mice exhibit growth retardation, high-frequency hearing loss, reduced thyroid activity, reduced body temperature, and some behavioral abnormalities (Grüneberg, 1965, 1971a,b). Hemizygous XO (*Eda^{Ta}*/O) females can be distinguished from their XX littermates on the basis of coat color patterns, and are fertile (Probst et al., 2008). Crosses of *Eda^{Ta}*/O females to *Eda^{Ta}*/Y males yield a frequency of *Eda^{Ta}* XO offspring from 10 to 20% (Probst et al., 2008).

Bone marrow preparation and fluorescence in situ *hybridization.* Bone marrow was extracted when the mice were killed from femurs and tibiae of representative XO, XX, and XY mice per the The Jackson Laboratory's protocol (http://www.jax.org/cyto/marrow_preps_alt.html). Mouse X and Y chromosome-specific probes were obtained from STARFISH (Cambio), and fluorescence in situ hybridization (FISH) was performed on bone marrow spreads as previously described (Probst et al., 2008).

Ovariectomy and hormonal manipulation. Ovaries were surgically removed 14 d before MCAO and mice were implanted with a subcutaneous SILASTIC capsule (0.062 inch inner diameter; 0.125 inch outer diameter) filled with either 0.035 ml of 17β-estradiol (180 μg/ml; Sigma) in sesame oil or oil vehicle as described previously (McCullough et al., 2005). Uterine weights were recorded when the mice were killed to confirm loss of estrogenic effects, and serum 17β-estradiol (E2) was measured by ELISA (IBL Hamburg).

Ischemic model. Cerebral ischemia was induced by either 60 or 90 min of reversible middle cerebral artery occlusion (MCAO, 20–25 g mice 10–12 weeks of age) under isoflurane anesthesia, followed by reperfusion, as previously described (McCullough et al., 2005). A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a 6.0 Doccol monofilament into the right internal carotid artery 6 mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. Sham-operated animals underwent the same surgical procedure, but the suture was not advanced into the internal carotid artery. Rectal muscle temperatures were maintained between 36.5 and 37.5°C during surgery and ischemia with an automated temperature control feedback system. In a separate nonsurvival cohort of animals, laser Doppler flow, arterial blood gases, and mean arterial pressure were monitored to ensure consistency of occlusion and equivalency of physiological variables between groups.

Behavioral scoring. Neurological deficits were scored 1 h after initiation of MCAO and when the mice were killed at 24 or 72 h after stroke. The scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the affected side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling (Li et al., 2004).

Histological assessment. Mice were killed at either 24 or 72 h after the onset of MCAO for infarct volume analysis. Brains were sectioned into five 2 mm slices, then stained with 2,3,5-triphenyltetrazolium chloride for 8 min at 37°C, followed by fixation in 4% paraformaldehyde. After digitalization of images, infarct volume was assessed using SigmaScan Pro (SPSS) as previously described (McCullough et al., 2005).

Statistical analysis. Data from individual experiments are presented as mean ± SEM. One-way ANOVA (with Tukey *post hoc* correction, when appropriate) was used for the comparison of the mean infarct volume between the experimental groups. Nonparametric results were analyzed with the Mann–Whitney *U* test. $p < 0.05$ was considered statistically significant. Due to the differences in coat phenotype among the mice investigators were blinded to genotype after removal of the brain for infarct analysis. Power analysis was performed on all groups to confirm appropriateness of sample size (G power).
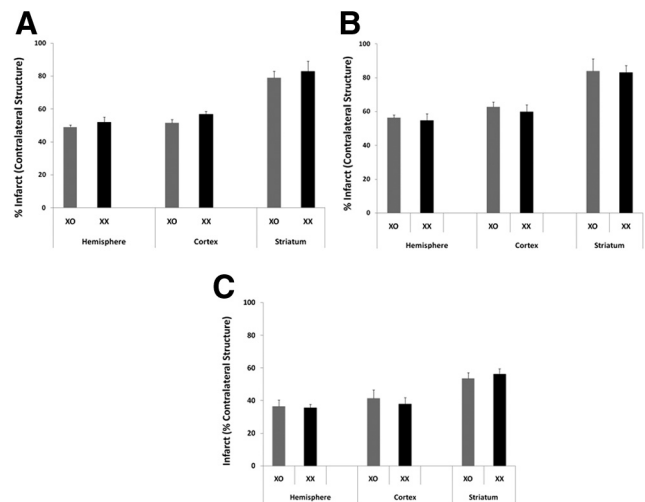

Figure 1. ***A***, After a 90 min occlusion, infarct sizes at 24 h after stroke are similar between ovariectomized Paf XX and XO females (*n* = 8 per genotype, respectively). ***B***, After 60 min of occlusion, at 72 h after stroke survival, there are no differences in infarct size between ovariectomized Paf XX and XO females (*n* = 7 per genotype, respectively). ***C***, X chromosome monosomy versus disomy has no effect on infarct size in estrogen-supplemented ovariectomized Paf XX and XO females (*n* = 7 per genotype, respectively) at 24 h after a 90 min occlusion.

Results

The ischemic sensitivity of *Paf* XO mice, which have loss of the paternally derived X chromosome, and their *Paf* XX littermates was compared. To confirm our coat phenotyping, we verified the X chromosome dosage in representative *Paf* XO and *Paf* XX females by sex chromosome-specific FISH. Baseline physiological parameters were similar between XO and XX females (data not shown). To eliminate the activational effects of ovarian hormones, a cohort of *Paf* XO and *Paf* XX littermates were ovariectomized 14 d before MCAO. With 90 min of ischemia, no differences were found in infarct size at 24 h after stroke between ovariectomized *Paf* XO and *Paf* XX females (hemisphere: 51% vs 49%; SEM ±4% vs ±3%; *n* = 8, *p* > 0.05) (Fig. 1*A*). To confirm that a ceiling effect had not been reached, and that differences would not emerge in infarct size with longer-term survival, we performed additional studies with a less severe ischemic insult (60 min) and a 72 h survival. Again no differences were observed between Paf XO and Paf XX ovariectomized females (hemisphere: 55% vs 57%; SEM ±1 vs ±4; *n* = 7, *p* > 0.05) after 72 h of ischemia (Fig. 1*B*). To confirm that *Paf* XO and *Paf* XX females were responsive to estrogen, a separate cohort of mice was supplemented with estrogen and subjected to a 90 min stroke with 24 h survival. As expected, estrogen levels were increased in supplemented animals (*Paf* XO Ovx+E2 estrogen levels = 41.6 ± 4 pg/ml; *Paf* XX Ovx+E2 estrogen levels = 37.3 ± 2 pg/ml; *p* > 0.05). Infarct size was not significantly different between *Paf* XO and *Paf* XX (hemisphere: 35% vs 36%; SEM ±4% vs ±4%; *n* = 7, *p* > 0.05, Fig. 1*C*), and was similar to gonadally intact *Paf* XO and *Paf* XX mice and significantly smaller than ovariectomized *Paf* XO and *Paf* XX females (as seen in Fig. 1*A*). As predicted, ovariectomized females of both groups had very low estrogen levels (Ovx XX estrogen = 9 ± 2 pg/ml; Ovx XO estrogen = 6 ± 2 pg/ml; *p* > 0.05) and atrophic uteri [Fig. 2*A*; Ovx XX uterine weight (UW) = 1.6 ± 0. mg/g of body weight (BW); Ovx XO UW = 1.7 ± 0.4 mg/g of BW; *p* > 0.05]. Neurobehavioral deficits were identical between all ovariectomized XX and XO cohorts, both during ischemia and at 24 h (Fig. 2*B*).
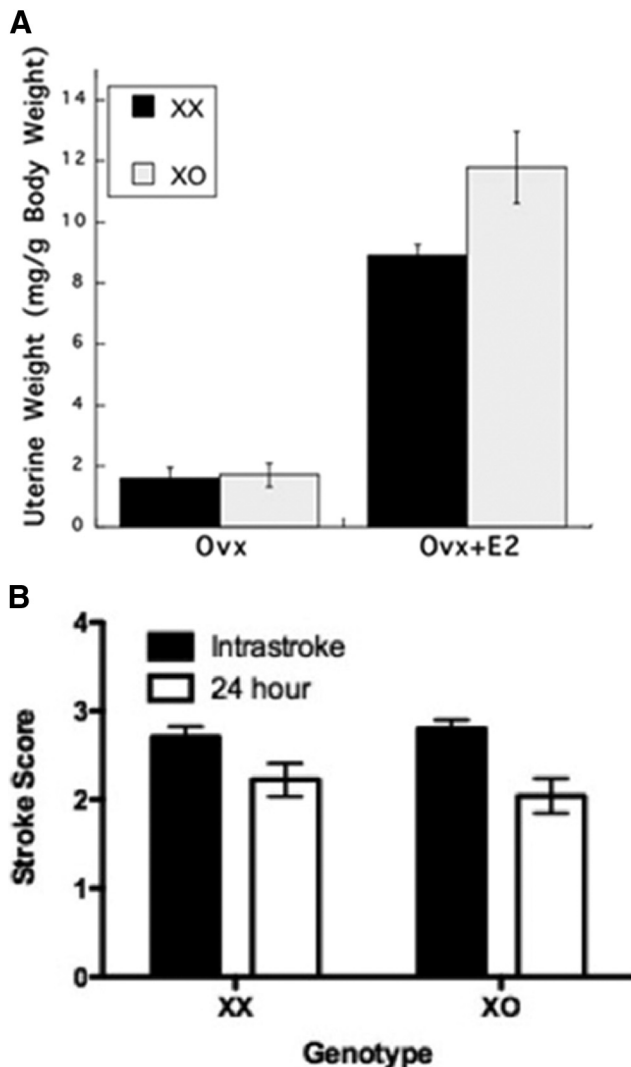
A

B

Figure 2. XX and XO mice display similar responses to hormone loss via ovariectomy and in the neurobehavioral deficits induced by stroke. *A*, Uterine weights in XX and XO mice, expressed in milligrams of uterine weight per gram of body weight. Uterine atrophy occurs to a similar extent in ovariectomized XX and XO mice. The effects of uterine atrophy are reversed by estrogen supplementation in ovariectomized XX and XO mice. *B*, Neurobehavioral scores are similar between XX and XO mice after 90 min of ischemia during both intraischemic and 24 h time points (XX: intrastroke = 2.7 ± 0.1; 24 h = 2.2 ± 00.2; XO: intrastroke = 2.8 ± 0.1; 24 h = 2.0 ± 0.2; $p > 0.05$).

We then examined gonadally intact *Paf* XO and *Paf* XX female littermates at 24 h after a 90 min cerebral occlusion to determine whether endogenous ovarian hormones (including progesterone, which is also lost with ovariectomy) interact with chromosome complement to influence ischemic outcome. No differences in ischemic sensitivity were observed in gonadally intact *Paf* XO and *Paf* XX female littermates at 24 h after a 90 min cerebral occlusion. There were no significant differences in infarct size between *Paf* XO and *Paf* XX intact females (hemisphere: 41% vs 45%; SEM ±6% vs ±6%; $n = 9$, $p > 0.05$) (Fig. 3). Infarct size was slightly higher in gonadally intact females than in ovariectomized females supplemented with estrogen. This was not statistically significant and was seen in both XO and XX mice, and likely represents the fluctuations in infarct size seen throughout normal estrous (Carswell et al., 2000).

To reduce the likelihood that there were mutation-specific or imprinting effects confounding interpretation of X chromosome
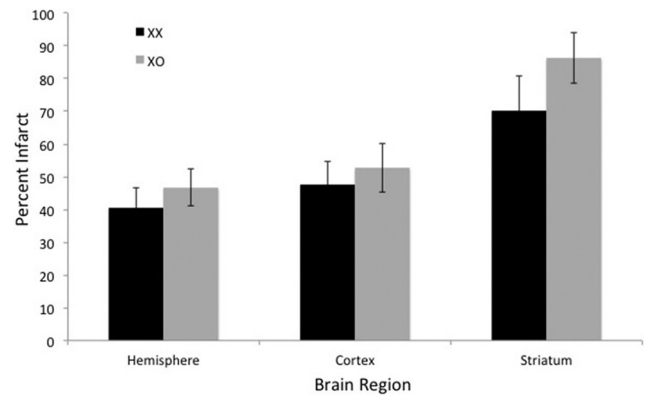

Figure 3. Infarct sizes are similar at 24 h after a 90 min occlusion in Paf XX and XO female mice with intact ovaries and normally cycling sex steroid hormones ($n = 9$ per genotype).
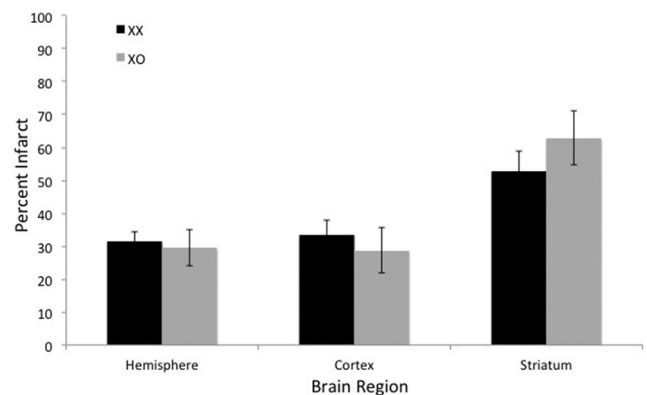

Figure 4. X chromosome monosomy versus disomy has no effect on infarct size in estrogen-supplemented ovariectomized Eda^{Ta} XX versus Eda^{Ta} XO females at 24 h after a 90 min occlusion ($n = 9$ per genotype). The smaller infarct sizes seen in the estrogen-supplemented group demonstrates that both XX and XO ovariectomized females are responsive to the neuroprotective effects of estrogen.

dosage, a second strain of XO mice (Eda^{Ta}/O), which have loss of the maternally derived X chromosome, was examined. To control for hormonal variations in infarct size Eda^{Ta}/O and Eda^{Ta}/+ females were ovariectomized and supplemented with estrogen. This also allowed us to compare infarct size of Eda^{Ta}/O with estrogen-treated Paf mice. There were no significant differences in infarct size at 24 h in Eda^{Ta} XO versus XX females (hemisphere: 31% vs 29%; SEM ±3% vs ±6%; $n = 9$; $p > 0.05$) (Fig. 4); estrogen levels (Ovx+E2 XX estrogen levels = 28 ± 2 pg/ml; Ovx+E2 XO estrogen levels = 33 ± 2 pg/ml; $p > 0.05$) and uterine weights were similar between groups (Fig. 2; Ovx+E2 XX UW = 8.9 ± 0.7 mg/g of BW; Ovx+E2 XO UW = 11 ± 1 mg/g of BW; $p < 0.05$). Infarct size was smaller in intact and in estrogen-treated mice than in ovariectomized mice, demonstrating that XO mice are responsive to estrogen.

Discussion

This is the first preclinical study to investigate the role of sex chromosome dosage in the response to stroke. No statistically significant differences in infarct size at 24 or 72 h after stroke were seen between two different strains of XO and XX mice, and this was independent of ovarian hormone status and the duration of ischemia. A strength of the present study is that two strains of XO mice with X chromosomes originating from different parent of origin were investigated. This allows preliminary investigation of the effects of X chromosome imprinting in stroke. The null chro-

mosome originates from the father in the *Paf* XO, resulting in an X chromosome of maternal origin (Lane and Davisson, 1990). In the *Eda^{Ta}* XO mouse, the colony breeding strategy ensures that the normal X chromosome is paternal (Probst et al., 2008). No difference in infarct size in comparison to XX littermates was seen in gonadally intact XO mice with an X chromosome of either parent of origin, suggesting that X chromosome imprinting effects are either not present or below the limits of detection of the present study.

While this investigation showed no overall effect of X chromosome dosage or parental imprinting on the response to cerebral ischemia in this model, these results do not preclude the possibility that individual X-linked genes are important in the ischemic response in humans. While one of the X chromosomes in females is randomly inactivated during early development, up to 20% of X-linked genes escape X inactivation in humans (Carrel et al., 1999). In contrast, a much smaller percentage of X-inactivation escapees exist in mouse (Tsuchiya and Willard, 2000; Disteche et al., 2002). In addition, homologs of some genes present on the human X chromosome are located on autosomes in mouse (Carrel et al., 1999; Tsuchiya and Willard, 2000; Disteche et al., 2002). For these genes, escape from X inactivation may be crucial for dosage in humans, but irrelevant in mouse. In addition, which genes escape from X inactivation can vary in a tissue-specific manner (Lopes et al., 2010). Whether a stressor, such as cerebral ischemia, can trigger expression of X-inactivation escapees in humans or in mice has yet to be investigated.

There are several limitations to these studies. These results contrast with clinical studies of human X chromosome monosomy, in which women with Turner syndrome experience an almost threefold higher risk of cerebrovascular diseases than in XX women (Gravholt et al., 1998; Gravholt, 2005; Stochholm et al., 2006; Schoemaker et al., 2008). Although the XO mice studied here represent murine X chromosome monosomy, they are an imperfect model of human Turner syndrome. In contrast to XO women, who experience premature ovarian failure if not given supplemental hormones (Gravholt, 2001), *Paf* and *Eda^{Ta}* XO mice are fertile. This difference in ovarian function may explain the divergent outcomes in stroke sensitivity.

A relatively severe insult, with 90 min of ischemia followed by reperfusion, was initially used, which may have led to a ceiling effect, in which maximal damage has occurred, making it difficult to observe more subtle effects from X chromosome loss. To address this limitation, and to confirm that the infarct size remained equivalent between the XO and XX mice after a longer survival, the effect of a milder insult of 60 min of ischemia with 72 h survival was investigated. No significant differences were observed between XX and XO mice after the milder injury, either. As XO and XX females show no differences in lesion volume after mild or severe ischemia, the contribution of X chromosome dosage to the early effects of cerebral ischemia in mice is likely to be minimal. Importantly, only young adult mice were examined in this study, and thus the current investigation does not exclude the possibility that changes could occur with age, especially as X inactivation can become unstable with aging (Hatakeyama et al., 2004; Smrt et al., 2011).

In these studies, mice were ovariectomized as adults to eliminate the activational effects of ovarian hormones; however, mice had been exposed to ovarian hormones until the time of ovariectomy, which does not eliminate the organizational effects of ovarian hormones. The influence of X chromosome dosage on ischemic sensitivity is likely small and not easily seen in a system with long-standing exposure to the organizational effects of ste-

roid hormones. Investigation of X chromosome loss in neonatal hypoxia–ischemia models may prove to be more informative, as mice of that age will not have experienced the surge of ovarian hormones at reproductive maturity. Likewise, studies in which mice are gonadectomized before puberty, then subjected to experimental stroke as adults, may be a better system in which to reduce the long-term organizational effects of sex steroid hormones.

While this is the first investigation to consider the role of intrinsic sex chromosome dosage in the response to cerebral ischemia, the present study leaves much unexplored. A recent study reported sex-specific parent of origin imprinting effects in the mouse brain, observed for both autosomal genes and the X chromosome (Gregg et al., 2010). Differential expression of these imprinting effects was observed in various brain regions. Whether or not the Y chromosome itself confers any increase or decrease in ischemic sensitivity is unknown. The presence of at least one X chromosome is crucial for rodent and human viability (Migeon, 2007), making an investigation of males with a YO phenotype impossible. An alternative approach could investigate the effects of cerebral ischemia in the XXY mouse model of Klinefelter syndrome (Lue et al., 2001), in which one of the two X chromosomes is thought to be randomly inactivated as in females to achieve dosage compensation between the sexes (Tüttelmann and Gromoll, 2010). Use of the four core genotypes model, in which gonadal sex can be separate from genetic sex (Becker et al., 2005; Arnold, 2009; Arnold and Chen, 2009), may also be a useful tool in further exploration of the role of the sex chromosomes themselves in stroke sensitivity.

In conclusion, X chromosome monosomy versus disomy does not significantly affect the degree of infarction in female mice either kept gonadally intact or ovariectomized as young adults.

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